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**PRIMING THE IMMUNE SYSTEM OF
LITOPENAEUS VANNAMEI WITH BACTERIAL
HEAT SHOCK PROTEIN 70 HOMOLOGUE
DNAK AGAINST *VIBRIO CAMPBELLII* AND
WHITE SPOT SYNDROME VIRUS (WSSV)
INFECTION**

BING HU

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD)
in Applied Biological Sciences

Dutch translation of the title: Activatie van het immuun systeem van *Litopenaeus vannamei* met het bacteriële hiteschockproteïne 70 homoloog DnaK tegen *Vibrio campbellii* en White Spot Syndrome Virus (WSSV) infectie

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Cover page: *Sicyonella* aff. *maldivensis* Borradaile, 1910. Photo by Tin-Yam Chan. (<http://www.zoologischemededelingen.nl/>)

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LIST OF ABBREVIATIONS AND UNITS

APC	Antigen presenting cell
ANOVA	Analysis of variance
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
°C	Degree Celsius
DAB	Diaminobenzidinetetrahydrochloride dehydrate
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EU	Endotoxin unit
FAO	Food and Agricultural Organization of the United Nations
FASW	Filtered and autoclaved seawater
g	Relative centrifugal force or G force
g/L	Gram per liter
GenBank	Genetic sequence database of the National Institute of Health, USA
h	Hour
HSC	Heat shock cognate
HSE	Heat shock elements
HSF	Heat shock factor
HSPs	Heat shock proteins
IFN	Interferon
IL	Interleukin
IU	International units
kDa	kilo Dalton

Kg	Kilogram
LB	Luria-Bertani
LPS	Lipopolysaccharides
M	Molar
NADPH	Nicotinamide adenine dinucleotide phosphate
MHC	Major histocompatibility complex
ng	Nanogram
NLHS	Non-lethal heat shock
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
P	Statistical p-value obtained
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Measure of the acidity of a solution
ppt	Parts per thousand
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real time-polymerase chain reaction
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TLR	Toll like receptor
TNF	Tumor necrosis factor
US\$	United States currency (Dollar)
v/v	Volume per volume
%	Percentage
μg	Microgram
μm	Micrometer

μL	Microliter
/	Per

CHAPTER 1

INTRODUCTION AND OBJECTIVE

Chapter 1

1. General introduction

1.1 Global scenario of aquaculture

Capture fisheries and aquaculture supplied the world with about 148 million tons of fish, crustaceans and molluscs in 2010, of which about 128 million tons were utilized as food. Preliminary data for 2011 indicate increased fish production to 154 million tons (FAO, 2012). The production of capture fisheries has leveled off since the 1990s (**Fig. 1.1**). The per capita consumption of seafood is increasing very fast because of the consumer awareness of health and nutritional benefits derived from dietary seafood in the diet, a good source of animal protein available at reasonable price. However, over-fishing is claimed to put marine ecology in danger (FAO, 2012) hampering further sustainable exploitation. To meet the consumer's demand, aquaculture became by and large the fastest growing food production industry.

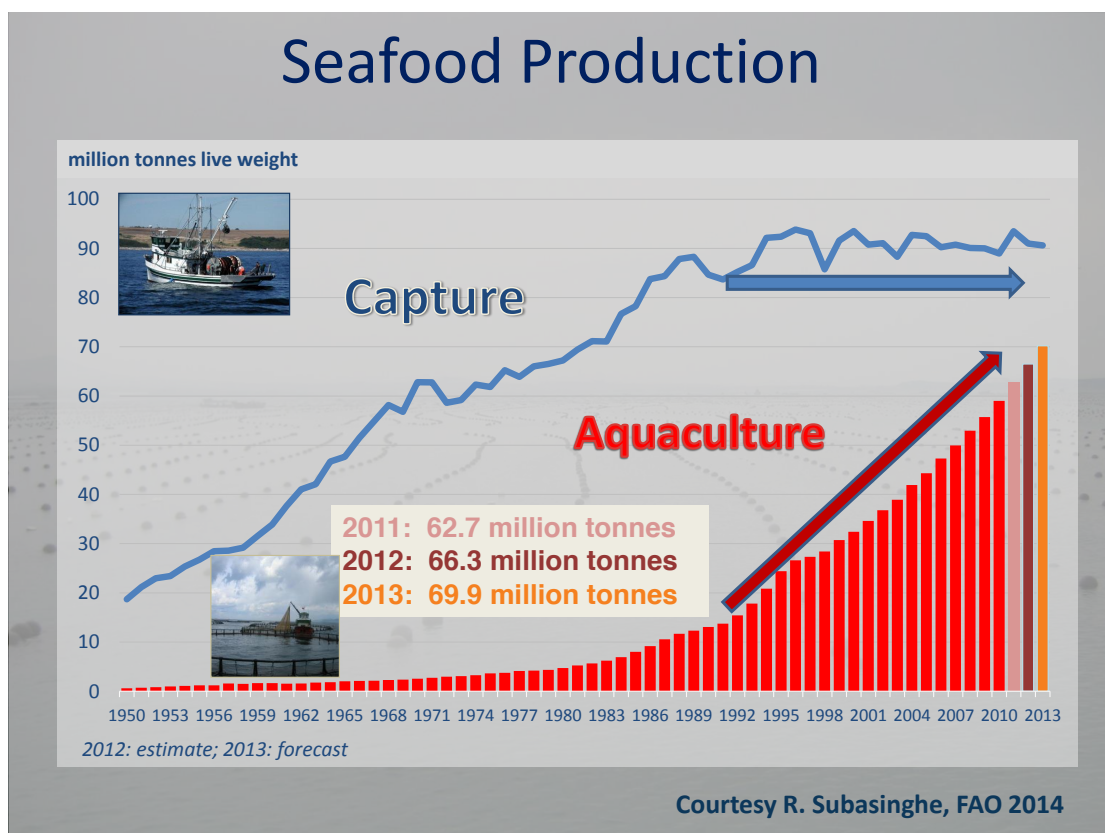


Figure 1.1 World capture fisheries and aquaculture production.

Global aquaculture production has continued to grow in the new millennium, albeit more slowly than in the 1980s and 1990s. World aquaculture production

attained another all-time high in 2010, at 60 million tons (excluding aquatic plants and non-food products) with an estimated total value of US \$ 119 billion (FAO, 2012). The global distribution of aquaculture production across the regions and countries of different economic development levels remains imbalanced (**Table 1.1**). In 2010, the top ten producing countries (China, India, Viet Nam, Indonesia, Bangladesh, Thailand, Norway, Egypt, Myanmar and Philippines) accounted for 87.6 percent by quantity and 81.9 percent by value of the world's farmed food fish. Asia accounted for 89 percent of world aquaculture production by volume in 2010, and this was dominated by the contribution of China which accounted for more than 60 percent of global aquaculture production volume. Other major producers in Asia are India, Viet Nam, Indonesia, Bangladesh, Thailand, Myanmar, the Philippines and Japan (FAO, 2012).

Table 1.1: Aquaculture production by region in 2010: quantity (tons) and percentage of world production (FAO, 2012)

Regions	Quantity (Million tons)	Percentage (%)
Africa	1 288 320	2.2
America	2 576 428	4.3
Asia	49 538 019	88.9
China	36 734 215	61.4
Europe	2 523 179	4.2
Oceania	183 516	0.3
World	59 872 600	

About 600 aquatic species are raised in captivity worldwide for production in a variety of farming systems and facilities of varying input intensities and technological sophistication, using freshwater, brackish water and marine water. In 2010, the composition of world aquaculture production was (**Fig. 1.2**): freshwater fishes (56.4 percent, 33.7 million tons), molluscs (23.6 percent, 14.2 million tons), crustaceans (9.6 percent, 5.7 million tons), diadromous fishes (6.0

percent, 3.6 million tons), marine fishes (3.1 percent, 1.8 million tons) and other aquatic animals (1.4 percent, 814 300 tons) (FAO, 2012).

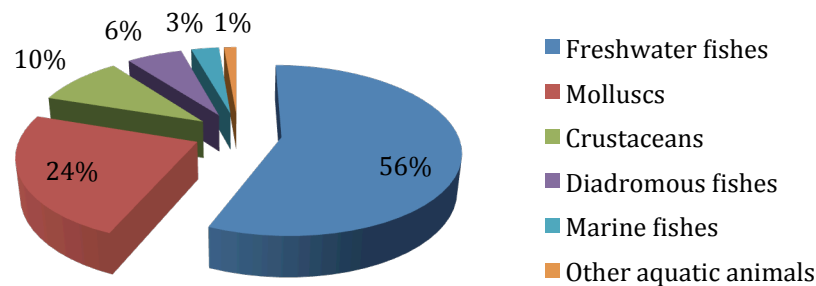


Figure 1.2 World aquaculture production: major species groups (FAO, 2012)

1.2 Disease outbreak in aquaculture

Aquaculture production is vulnerable to adverse impacts caused by disease and environmental conditions. Disease outbreaks in recent years have affected farmed Atlantic salmon in Chile, oysters in Europe, and marine shrimp farming in several countries in Asia, South America and Africa, resulting in partial or total loss of production. In 2010, aquaculture in China suffered production losses of 1.7 million tons caused by natural disasters, diseases and pollution. Disease outbreaks virtually wiped out marine shrimp farming production in Mozambique in 2011 (FAO, 2012). The aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens (Bondad-Reantaso et al., 2005). Several major microbial pathogens causing infectious diseases have been identified until now (Austin and Zhang, 2006). Among those microbial pathogens, *Vibrio* spp. and White Spot Syndrome Virus (WSSV) are by far the most aggressive, causing massive mortalities of either fish or shellfish in worldwide culture (Austin and Zhang, 2006; Chen et al., 2000; Escobedo-Bonilla et al., 2008).

Vibrio diseases are commonly described as “vibriosis” which are caused by several species of Gram-negative bacteria such as *Vibrio campbellii*, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, etc. Typically found in saltwater, *Vibrio* spp. are motile and have polar flagella with sheaths. In fish,

clinical signs of vibriosis include weight loss, lethargy, red spots on the swollen ventral and lateral areas of the fish and dark skin lesions that can ulcerate, and bleed. In crustaceans, bioluminescence and melanization are also signs of vibriosis (**Fig. 1.3**).

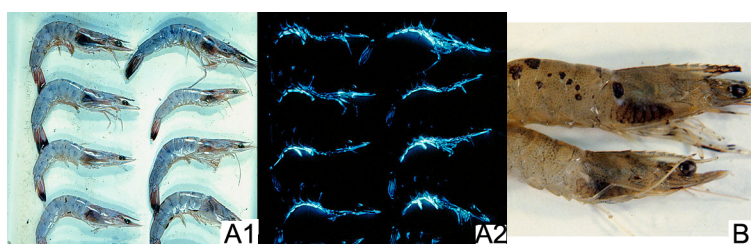


Figure 1.3 Luminescent vibriosis (A1, A2) and melanization (B) in shrimp. (Source: http://mail-cenaim.espol.edu.ec/noti/cursos_material/curso19/ligthner/Photo4_2.htm)

Apart from bacterial infections, viral infections have been threatening aquaculture development for decades. Among these viruses, white spot syndrome virus (WSSV) is one of the most intensively studied viruses which has been characterized from aquatic animals especially crustaceans (Flegel, 2006). Infection with WSSV has caused detrimental diseases in shrimp culturing, resulting in serious economic losses (Dhar et al., 2004).

WSSV is an enveloped large circular double stranded DNA virus containing about 300Kbp (Van Hulten et al., 2001). The enveloped virions (120–150 nm, 270–290 nm) are symmetrical and ellipsoid to bacilliform in shape similar to baculovirus (**Fig. 1.4**). Phylogenetic analysis suggests that WSSV belongs to a new virus family named as the Nimaviridae, genus Whispovirus (Vlak et al., 2002). White Spot Syndrome Virus has been the most problematic infectious agent affecting the global shrimp farming industry since the emerging of the disease in 1992. The disease was named after its primary clinical sign in affected *Penaeid monodon*: formation of circular white calcium deposits on the underside of the cuticle of the cephalothorax. In infected American penaeids, whose WSSV denomination can be misleading, the virus rarely induces white spots as similar spotting of the cuticle may result from other causes, such as bacterial infections (Wang et al., 2000). The WSSV has a wide host range including all species of

shrimps, some other crustaceans (e.g. crayfish, crab, and spiny lobster), and has even been detected in insects.

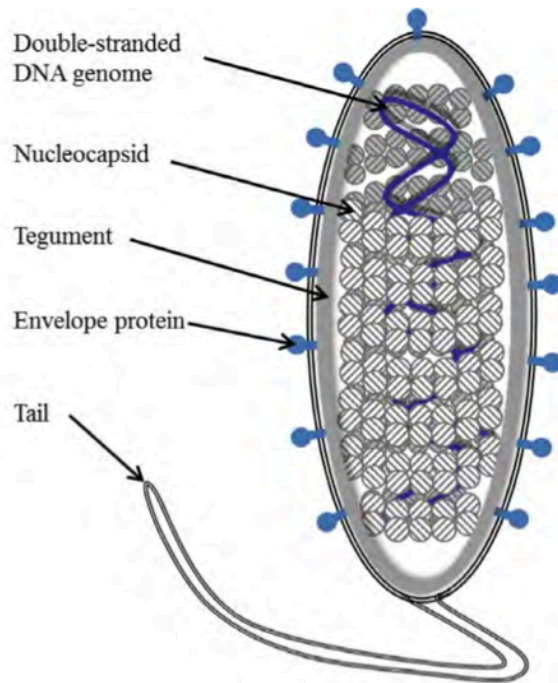


Figure 1.4: Schematic representation of the White Spot Syndrome Virus particle (Corteel, 2013).

1.3 Aquatic animal health management

Conventional therapy using antibiotics aiming at eliminating pathogens has been found detrimental to human health and environment. Abuse of antibiotics can result in antibiotic-resistant strains, hampering further use of antibiotics. Based on such concerns, the European Union imposed a ban on antibiotic growth promoters in the animal production sector in 2006. More and more countries and regions were realizing the threat related to the misuse of antibiotics. To control aquaculture diseases for a sustainable development, alternative disease control techniques are urgently needed. However, our knowledge of health control in aquatic animals remains very limited, especially in invertebrates: crustaceans and molluscs. Much more basic work, including the use of molecular tools, should improve our knowledge on the good functioning of the animal's immune systems, including ways to activate or inactivate it. Only then it will be

possible to move from empirical trial and error practice today – with all kinds of putative immune-stimulants and probiotics – to knowledge-based strategies and products.

2. Objectives and thesis outline

Historically, many anti-infective strategies replacing antibiotics have been tested such as green water techniques, immunostimulants, probiotics, bacterial quorum sensing (Defoirdt, 2011; Harikrishnan et al., 2011; Ninawe and Selvin, 2009; Papandroulakis et al., 2001). Those anti-infective strategies have shown significant protective effect, but none of them is generally applied throughout the aquaculture industry, most probably because of the variability between aquaculture species. There is increasing need for anti-infectious techniques that can be applied in a broad range of aquatic species and aquaculture environment.

The **general objective of this thesis** is to evaluate a novel anti-infective strategy to control vibriosis and WSSV in aquaculture. More specifically, investigations were performed to elucidate the role of recombinant heat shock protein 70 (Hsp 70), derived from a prokaryotic source, in generating an immune response (**Chapter 3 and 4**) and to instigate protection against pathogenic *Vibrio campbellii* (**Chapter 5**) and White Spot Syndrome virus (WSSV) (**Chapter 6**).

Heat shock proteins (HSPs), also known as stress proteins and extrinsic chaperones, are a suite of highly conserved proteins of varying molecular weight (c. 16–100 kDa) produced in all cellular organisms when they are exposed to stress (Roberts et al., 2010). They play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families, such as HSP90 and HSP70, are critical for the folding and assembly of other cellular proteins and are also involved in regulation of kinetic partitioning between folding, translocation and aggregation within the cell. HSPs also have a wider role in relation to the function of the immune system, apoptosis and various facets of the inflammatory process (Guo et al., 2013; Jolesch et al., 2012; Loc et al., 2013). In aquatic animals, they have also been shown to play an important role in health, in relation to the host response to environmental pollutants, to food toxins and in particular in the development of inflammation and the specific and non-specific immune

responses to bacterial and viral infections in both finfish and shrimp (Baruah et al., 2010; Deane and Woo, 2005; Sung et al., 2009). However, the mechanism underlying it is still ambiguous, especially in invertebrates (Roberts et al., 2010).

This dissertation starts with a literature review summarizing current knowledge of crustaceans immunity and heat shock proteins with a main focus on the HSP70-induced immune response in innate immunity (**Chapter 2**). Chapter 2 is followed by four chapters with experimental data showing the immune regulatory ability and priming effect of bacterial HSP70 (DnaK) (**Chapters 3-6**). Finally, the experimental findings are summarized and discussed in **Chapter 7** and perspectives are raised.

➤ **Chapter 2** (Literature overview) consists of two parts: Part I introduces the role of *Litopenaeus vannamei* and immune characters of shrimps. It summarizes the research progress made on the immune defense mechanism in crustaceans especially in shrimps. Part II gives an overview of the current knowledge on the immunogenicity and protective efficacy of HSPs against microbial diseases. As crustaceans lack an adaptive immune response HSP70 involvement in the innate immunity response is mainly discussed.

➤ In **Chapter 3**, the role of bacterial HSP70 (DnaK) as an efficient immuno-stimulant in *L. vannamei* was verified by including a chemically synthetic, microbial contaminants free DnaK fragment peptide (DnaK₄₄₂₋₄₉₁) as control. The data on the induction of the two immune genes described in chapter 3 were re-used in chapter 4 as they served a different research purpose.

➤ In **Chapter 4**, in order to evaluate the acute immune stimulatory effect of DnaK in *L. vannamei*, regulation of four important immune-related gene groups (prophenoloxidase, transglutaminase, penadins, endogenous HSP70) were monitored 12h after DnaK intramuscular injection. Significantly responding genes were selected as immune markers for subsequent study.

➤ In **Chapter 5** we tested the hypothesis that DnaK can prime the immune system of *L. vannamei* to cope with a subsequent bacterial

Chapter 1

infection (*Vibrio campbellii*).

➤ In **Chapter 6**, on the other hand, we tested the hypothesis that DnaK can prime the immune system of *L.vannamei* to cope with a viral infection (WSSV).

➤ **Chapter 7** recapitulates the overall findings obtained in the four experimental chapters above, conclusions are drawn and possibilities for future research are proposed.

CHAPTER 2

LITERATURE OVERVIEW

Part 1: *LITOPENAEUS VANNAMEI* AND SHRIMP IMMUNITY

1. *LITOPENAEUS VANNAMEI*

Litopenaeus vannamei is an important aquaculture species in the world. According to the statistics, it shared the largest proportion (more than 2.5 million tons) of crustaceans in aquaculture (FAO, 2012). *L. vannamei* belonging to the largest phylum in the animal kingdom, the Arthropod, is an invertebrate animal having an exoskeleton (external skeleton), a segmented body and jointed appendages. Under this phylum, *L. vannamei* resorts to the crustaceans which includes crabs, lobsters, crayfish, shrimp, krill and barnacles.

Table 2.1 Scientific classification of *L. vannamei*

Phylum	<i>Arthropoda</i>
Subphylum	<i>Crustacea</i>
Class	<i>Malacostraca</i>
Order	<i>Decapoda</i>
Suborder	<i>Dendrobranchiata</i>
Family	<i>Penaeidae</i>
Genus	<i>Litopenaeus</i>
Species	<i>L. vannamei</i>

L. vannamei grows to a maximum length of 230 millimeters (mm) with a carapace length of 90 mm. Adults live in the ocean, at depths of up to 72 meters, while juveniles live in estuaries. The rostrum is moderately long, with 7–10 teeth on the dorsal side and 2–4 teeth on the ventral side (Ruppert and Barnes, 1994). *L. vannamei* are native to the eastern Pacific Ocean, from the Mexican state of Sonora to as far south as northern Peru. It is restricted to areas where the water temperature remains above 20 °C throughout the year.

During the 20th century, *L. vannamei* was an important species for Mexican inshore fishermen, as well as for trawlers further offshore. In late 20th century, the wild fishery production was overtaken by aquaculture production; this began in 1973 in Florida using shrimp captured in Panama. In Latin America, the culture of *L. vannamei* showed peaks of production during the warm El Niño years, and reduced production during the cooler La Niña years, due to diseases. Production of *L. vannamei* is limited by its susceptibility to various diseases, including white spot syndrome, Taura syndrome, infectious hypodermal and haematopoietic necrosis, baculoviral midgut gland necrosis and *Vibrio* infections. By 2004, global production of *L. vannamei* approached 1,116,000 tons and exceeded that of *Penaeus monodon*. In 2012, *L. vannamei* production reached to 3,178,721 tones.

2. IMMUNE TRAITS

As for other crustaceans, the immune system of *L. vannamei* is well-known to be non-specific (innate), while increasing evidence implies that they might possess specific or adaptive immune responses as well (Hauton et al., 2007). The innate immune response is the first line of defense against microbial infections in both vertebrates and invertebrates (Hoffmann et al., 1999). It triggers diverse humoral and cellular activities *via* signal transduction pathways/cascades which are conserved both in insects and mammals (Borregaard et al., 2000). The immune responses are triggered *via* the surface receptors of immune cells. Diverse pathogen related immune cell surface receptors, pathogen recognition receptors (PPRs) have been identified. Parts of the immune cascades and signal transduction pathways, such as prophenoloxidase cascade, clotting cascade and Toll pathway etc. have been discovered and elucidated. In shrimp immunity, the blood cells (hemocytes) also play important role. They are crucial in these immune reactions and are capable of phagocytosis, encapsulation, nodule formation, and mediation of cytotoxicity.

Thus, the immunity of crustaceans is generally grouped into humoral and cellular immunity. The humoral immune responses include the immune cascades, signal transduction pathways and a wide array of antimicrobial peptides (AMPs). Compared with humoral immunity, studies on the processes that govern the

cellular immune response are very limited (Williams, 2007). The cellular response involves the hemocytes synthesis, phagocytosis, apoptosis, nodule formation and encapsulation of pathogens. Still, its defense mechanisms including the immune signal transaction pathways, are not fully clear until now. Due to the economic importance of shrimp farming, researchers have paid more attention to the mechanism of shrimp disease outbreak and tried to develop efficient control strategies. Therefore, a brief summary about the immune system of shrimp is reviewed based on the latest discoveries, which will help us to understand the immune responses of shrimp against different pathogens.

2.1 Pathogen recognition receptor (PRR)

Mackay et al. (2000) described pathogen recognition receptors (PRRs), as a set of germ line-encoded receptors as causing activation of the innate immune response involving the recognition of pathogens, different from vertebrate antibodies. Pathogen associated molecular patterns (PAMP) can be recognized by PRR. The recognition leads to rapid humoral and cellular immune responses. The generally accepted concept is that these PAMPs molecular patterns are usually the polysaccharides and glycoproteins on the surface of microbes, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells. The patterns can be polynucleotides, such as bacterial and viral unmethylated CpG (—C—phosphate—G—) DNA, single-strand and double-strand RNA from viruses (Christophides et al., 2004; Jensen and Thomsen, 2012). These molecular patterns are present not only in pathogens but in all microbes; thus the term “microbe-associated molecular patterns (MAMPs)” is now a new name used for all of them.

Additionally, highly specific invertebrate immune responses against specific pathogens were subsequently reported in the fruit fly *Drosophila melanogaster* (Watson et al., 2005) and the mosquito *Anopheles gambiae* (Dong et al., 2006). In light of all this new evidence, the innate immune responses of invertebrates are now thought to be supplemented by a unique immune mechanism with specificity and memory. In other words, the limited invertebrate adaptive immunity provides the same functionality as vertebrate adaptive immunity. The

mechanisms and molecules in invertebrates that support the production of antigen-specific receptors with high diversity were originally unknown, but recent reports have pointed to a receptor in the immunoglobulin superfamily (IgSF), the Down syndrome cell adhesion molecule (Dscam).

To date, 11 types of pattern recognition receptors (PRRs) have been identified in shrimp, with 6 types have been discovered in *L. vannamei* (**Table 2.2**), including β -1,3-glucanase-related proteins (BGRP), β -1,3-glucan-binding protein (BGBP), C-type lectins, thioester-containing proteins (TEPs), fibrinogen-related proteins (FREPs), scavenger receptors (SRs), Down syndrome cell adhesion molecules (DSCAMs) and Toll like receptors (TLRs) (X.-W. Wang and J.-X. Wang, 2013). Like other invertebrate PRRs, shrimp receptors have rather broad ligand-binding specificity, including LPS, LTA, β -glucan and proteins, but the molecular basis for this recognition is still poorly understood. Most likely new PRRs in will be identified shrimp immunity. For example, the NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) are very important PRRs in animals and plants (Maekawa et al., 2011), most animal NLRs are activated by PAMPs or endogenous substances released by damaged or dying cells, called damage-associated molecular patterns (DAMPs). At present, the relevant shrimp receptors have not yet been identified. In *Drosophila*, the recognition of pathogens by PGRPs or GGBP can induce intracellular signaling, resulting in the expression of effectors (Lemaitre and Hoffmann, 2007); the mammalian mannose-binding lectin can also stimulate the complement system upon recognition (Ip et al., 2009). However, few studies demonstrate that shrimp PRRs are linked to signal pathways and specific immune effectors. Therefore, finding downstream responses after pathogen recognition would be helpful in understanding the important functions of shrimp PRRs.

Table 2.2: Reported pattern recognition receptors in penaeid shrimp. (X.-W. Wang and J.-X. Wang, 2013a)

PRRs in <i>L.vannamei</i>		
PRRs	Receptor numbers	Reference
β -1,3-glucanase-related proteins (BGRP)	1	(Cheng et al., 2005)
β -1,3-glucan-binding protein (BGBP)	1	(Romo-Figueroa et al., 2004)
C-type lectins	7	(X. Wang and J. Wang, 2013b)
Thioester-containing proteins (TEP)	1	(Gollas-Galvan et al., 2003)
DSCAM	1	(Chou et al., 2009)
Toll like receptors (TLR)	3	(Wang HQ et al., 2010)
PRRs in other Penaeid shrimp species		
PRR	Species	References
Scavenger receptor (SCR)	<i>Metanephrops japonicus</i>	(Mekata et al., 2011)
Serine proteinase homologue (SPH)	<i>Penaeus monodon</i>	(Liu et al., 2011)
Trans-activation response RNA-binding protein (TRBP)	<i>Fenneropenaeus chinensis</i>	(Wang S. et al., 2012)

2.2 Immune signal transaction cascades/pathways

2.2.1 Prophenoloxidase (proPO) cascade

The prophenoloxidase cascade is also known as melanization, which is essential for microbial pathogen elimination in crustaceans (Söderhäll and Cerenius, 1998). The proPO system is a multistep pathway. The recognition of microbial PAMPs by PRPs leads to the activation of the serine proteinases (SPs) cascade then subsequently culminates in the proteolytic cleavage of the proPO

zymogen to the active phenoloxidase (PO) enzyme. The activated PO eventually catalyzes the oxidation of tyrosine-derived phenols to quinones believed to be directly toxic to micro-organisms, and to polymerize non-enzymatically to form insoluble melanin that physically encapsulates pathogens (Amparyup et al., 2013; Cerenius et al., 2008; Söderhäll and Cerenius, 1998).

In penaeid shrimp, parts of the proPO cascade have been elucidated in *Penaeus monodon* (**Fig. 2.1**). Initially, β -1,3-glucan binding protein (PmLGBP) plays a role in the recognition of microbes by acting as a functional pathogen recognition protein (PRP) for LPS and β -1,3-glucan detection. The PRP-PAMP complex then may initiate the clip-SP cascade that can convert the inactive shrimp prophenoloxidase-activating enzymes (PPAEs). The elicitation of PPAEs leads to the activation of the two proPOs (PmproPO1 and PmproPO2), subsequently resulting in the production of melanin and reactive oxygen compounds.

Interestingly, specific down-regulation of these two proPOs significantly decreased the gene expression levels of the two antimicrobial peptides (AMPs), PEN3 and Crus-likePm, and two genes in the proPO system PmLGBP and PmPPAE2, supporting a likely cross-talk between the proPO system and AMP gene synthesis in *P. monodon* (Amparyup et al., 2013). Moreover the cross-link between proPO cascade and clotting cascade has been suggested (Sritunyalucksana and Söderhäll, 2000).

2.2.2 Clotting cascade

Clotting is an important reaction aimed at preventing hemolymph loss and microbial spread at sites of injury. The clotting system is the first line of defense and an integral part of the overall invertebrate immune system. In crustaceans, the molecular mechanism of the clotting system was firstly unveiled in crayfish. A calcium-dependent transglutaminase (TGase), which is released from the hemocytes under foreign particle stimulus or tissue damage, catalyzes cross-linking aggregation of clotting proteins (CPs) (Sritunyalucksana and Söderhäll, 2000).

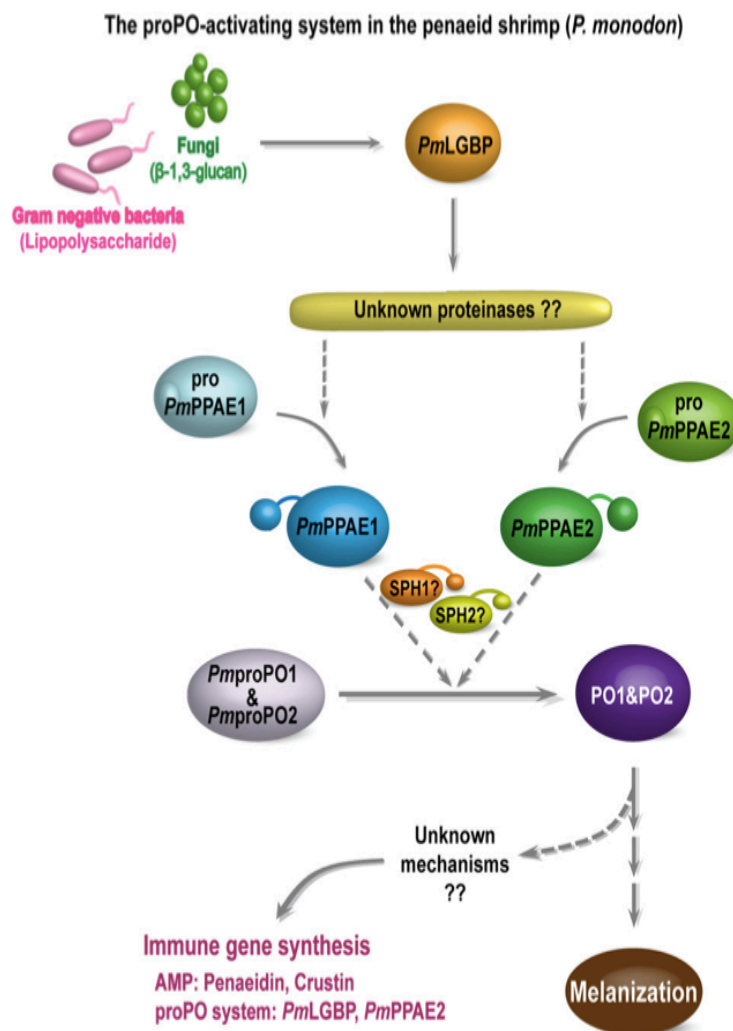


Figure 2.1 Proposed mechanism for the activation of the proPO cascade in the penaeid shrimp *P. monodon*. PmLGBP is a pattern-recognition protein (PRP); PmPPAE1 and PmPPAE2 are prophenoloxidase-activating enzymes (PPAEs); PmMasSPH1 and PmMasSPH2 are clip-domain serine proteinase homologs (Clip-SPHs) and PmproPO1 and PmproPO2 are prophenoloxidases (proPOs). (Amparyup et al., 2013)

The shrimp clotting system is an essential part of the shrimp immunity. In this clotting system, the central enzyme, TGase, was localized in the hemocytes of crustaceans (R. Wang et al., 2001) and found to be involved in cell proliferation (Lin et al., 2008) and immune response against bacterial and viral infection (Maningas et al., 2008). Besides, increasing amounts of studies have implicated TGase with multifunctional properties

in immune mechanisms in invertebrates, such as pathogen entrapment (Z. Wang et al., 2010), immune gene regulation (Fagutao et al., 2012), hematopoiesis (Lin et al., 2008; Lin and Söderhäll, 2011), cuticle morphogenesis and sclerotization (Shibata et al., 2010) etc. From all together, TGase is the most conserved protein in the coagulation system (Cerenius et al., 2010a; Jiang and Doolittle, 2003; Loof et al., 2011). Although the functions of TGase are becoming better understood, it is still unclear how TGase (or this protein) is released from the hemocytes in the presence of plasma clotting proteins.

2.2.3 Toll pathway

The Toll pathway plays a key role in the response to Gram-positive bacteria and fungi by regulating a large set of genes (including antimicrobial peptide genes, many small peptides with unknown function as well as components of the melanization and clotting cascades). The canonical component of the Toll pathway in *Drosophila* contains Spätzle, Toll, Pelle, Tube, MYD88, Cactus, Dorsal, Dorsal-related immunity factor (DIF) (Hoffmann and Reichhart, 2002; Hultmark, 2003; Lemaitre and Hoffmann, 2007) (**Fig. 2.2**). TRAF 6 was also documented to be a downstream molecule of Pelle in the Toll pathway of *Drosophila* (Aggarwal and Silverman, 2008). Some evidence showed that the Toll pathway was involved in the immune response to virus in *Drosophila* (Sabin et al., 2010; Zambon et al., 2005). In shrimp, most homologs to the components in Toll pathway of *Drosophila* have been identified, including Spätzle, Toll receptor, Pelle, TRAF6, Dorsal, and their function in the immune response of shrimp to bacteria or virus were supposed and deduced (Arts et al., 2007; F. Li et al., 2010; P.-H. Wang et al., 2011a; 2011b; Yang et al., 2008). Full-length cDNAs of MYD88 and Cactus were obtained recently, and further study on their function has been taken up (C. Li et al., 2012; Wen et al., 2013).

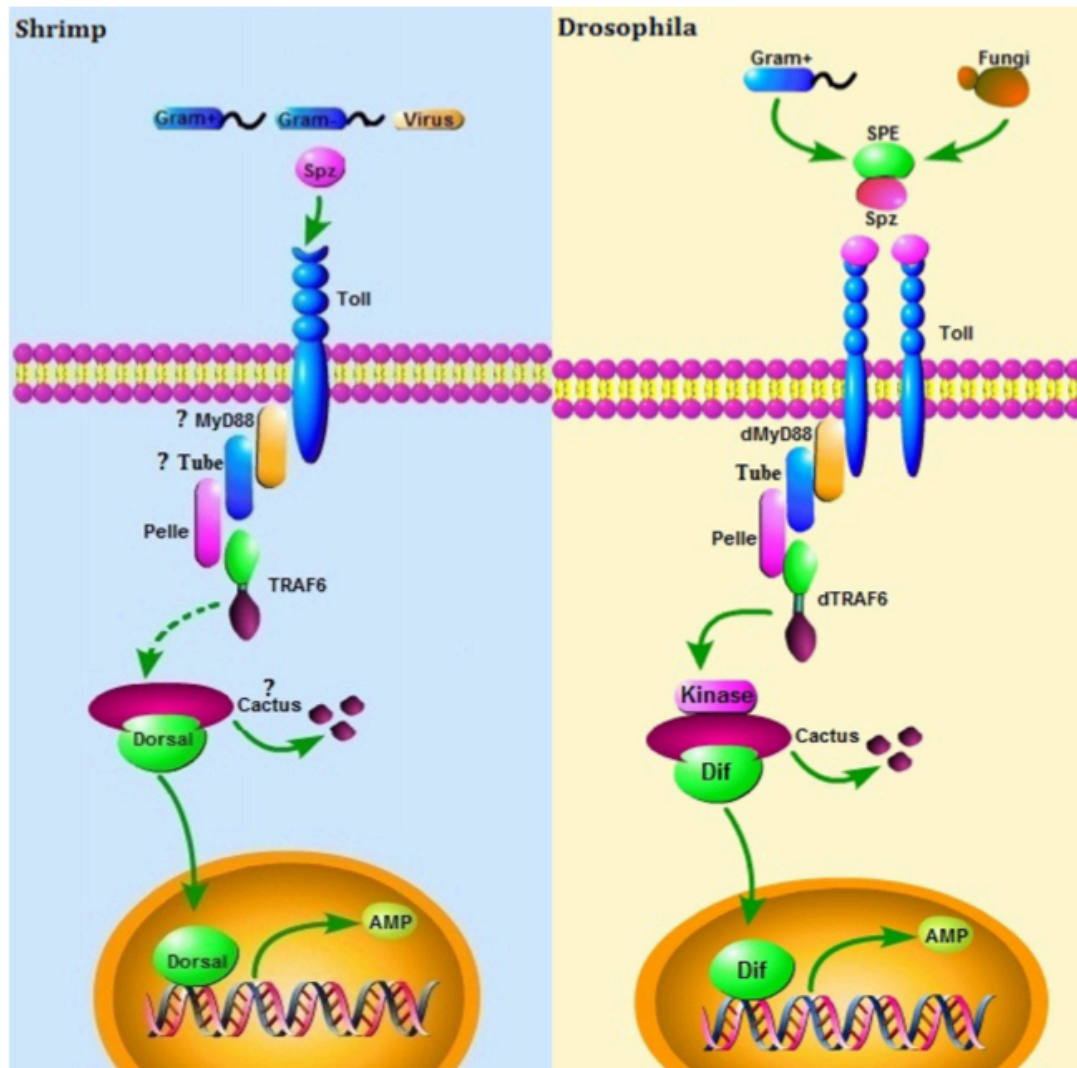


Figure 2.2 Comparison of Toll pathways in shrimp and *Drosophila*. (Li and Xiang, 2013)

2.2.4 The immune deficiency (IMD) pathway

Both the Toll pathway and the IMD pathway are important signaling cascades which regulate the expression of antimicrobial peptide genes in *Drosophila* (De Gregorio et al., 2002). The Toll pathway responds to Gram-positive bacterial and fungal pathogens, while the IMD pathway preferentially recognizes Gram-negative bacteria (Lemaitre and Hoffmann, 2007). The *Drosophila* IMD pathway includes 9 canonical components: IMD, the PGRP-LC receptor, the mitogen-activated protein 3 kinase (TAK1), TAB2, DIAP2 (a member of inhibitor of apoptosis proteins), IKK signalosome (complex of IKKb/ird5 and IKKg/Kenny),

the dFADD adaptor, the Dredd caspase, and the transcription factor Relish (Lemaitre and Hoffmann, 2007) (**Fig 2.3**). Up to date, knowledge on the role of IMD pathway in the immune response of shrimp is very limited, except for two identified components, IMD and Relish (Li et al., 2009; P.-H. Wang et al., 2009).

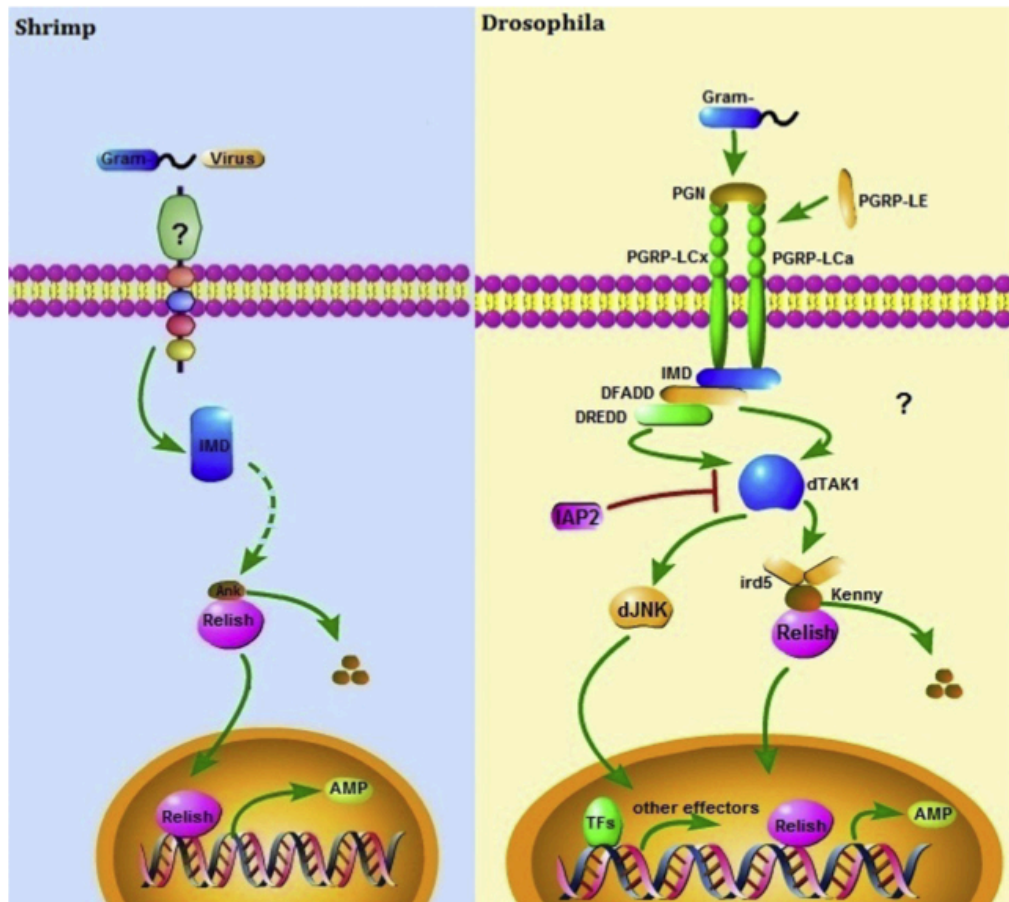


Figure 2.3 Comparison of IMD pathways in shrimp and *Drosophila*. (Li and Xiang, 2013)

2.2.5 The JAK/STAT pathway

The JAK/STAT (Jaus kinase/signal transducer and activator of transcription) pathway, including three main cellular components - the receptor Domeless, the Janus Kinase (JAK) Hopscotch and the STAT transcription factor - is involved in regulating the immune response genes (Agaisse and Perrimon, 2004). The JAK/STAT pathway has been implicated in antiviral defense in insects (Dostert et al., 2005; Sim and Dimopoulos, 2010; Souza-Neto et al., 2009). Chen et al. (2008) reported the first STAT homolog in *P. monodon* which had the typical functional domains, including the DNA binding domain, SH2 domain and C-terminal

transactivation domain which are conserved with those reported in insects (W.-Y. Chen et al., 2008). Sun et al. (2011) also isolated the full-length cDNA of STAT homologs from *F. chinensis* (Sun et al., 2011). The isolation of STAT in shrimp indicated that the JAK/STAT pathway might exist in shrimp. The transcription of STAT in shrimp was modulated after WSSV infection (W.-Y. Chen et al., 2008; Sun et al., 2011), which indicated that the JAK/STAT pathway might be very important in shrimp responsive to virus infection. In order to clarify the function of this pathway, other components need to be identified, and the interaction among these components are in urgent need for study.

2.3 Blood cells (hemocytes) and cellular immunity

2.3.1 Blood cells (hemocytes) in crustaceans and their role in immunity

In crustaceans, oxygen transport in the circulatory system is via oxygen transport pigments of the hemocyanin protein family, which are present in plasma. Cells involved in protecting the animal from invading organisms and wound healing are also involved in this circulatory system. Crustaceans only rely on very efficient innate immune mechanisms in which these blood cells (hemocytes) play a key role (Lin and Söderhäll, 2011). Apart from participating in immediate immune reactions such as clotting, melanization, phagocytosis, and encapsulation, hemocytes are important suppliers of different antimicrobial peptides, lectins, proteinase inhibitors and opsonins such as the cell adhesion protein peroxinectin (Cerenius et al., 2008).

Three main types of hemocytes can be identified in most decapod crustaceans: hyaline cells, semi-granular cells (SGCs), and granular cells (GCs); the hyaline cells are small and contain no granules, or very few, and may act as phagocytes. This cell type is common in marine crustaceans (Dantas-Lima et al., 2013; Roulston and Smith, 2011). The number and proportion of different hemocyte types varies a great deal among crustaceans and is influenced by various environmental conditions (Söderhäll et al., 2003). The SGC are usually the most abundant cell type, making up approximately 65% of the cells (Wu et al., 2008). This hemocyte type contains a variable number of small eosinophilic granules and is involved in early recognition, coagulation and, to some extent, phagocytosis. This is the main hemocyte type involved in encapsulation of

microorganisms. Encapsulation reactions are usually followed by melanization, and the SGCs contain the components of the proPO system. The expression of one specific kazal-type proteinase inhibitor can be used as a marker for the differentiation of stem cells along the SGC lineage.

However, the main repositories for the proPO system are the GCs, which are densely packed with large eosinophilic granules, and they release their contents by exocytosis on activation. Apart from the proPO system, the granules contain different antimicrobial peptides (AMPs), various proteinase inhibitors, and the cell adhesion/degranulating factor peroxinectin, a homologue of vertebrate myeloperoxidase (Cerenius et al., 2010b; Sricharoen et al., 2005). Superoxide dismutase is exclusively expressed by cells of the GC lineage and can be used to identify these cell types in crayfish (Wu et al., 2008).

2.3.2 Blood cell synthesis

Response to injury or infection leads to a dramatic loss of free circulating hemocytes, which is followed by a recovery phase accomplished mainly by rapid synthesis and release of new hemocytes from hematopoietic tissue (HPT) (Söderhäll et al., 2003). In decapod crustaceans, such as the shore crab *Carcinus maenas*, the lobster *Homarus americanus*, and the crayfish *Pacifastacus leniusculus*, HPT is composed of a series of ovoid lobules that collectively form a thin sheet on the dorsal part of the foregut (Johansson et al., 2000). The HPT of crayfish is penetrated by vessels and provides a site for stem cell renewal as well as for the development of mature hemocytes that are released into the circulation as a rapid response to peripheral needs (Söderhäll et al., 2003).

Cell proliferation in the HPT can be influenced by different stress factors such as moulting, lipopolysaccharide injection, and manganese exposure (Hammond and Smith, 2002; Hernroth et al., 2004). Their synthesis is regulated by the astakine cytokines (**Fig. 2.4**). Astakines are among the first invertebrate cytokines shown to be involved in hematopoiesis and they can stimulate the proliferation, differentiation and survival of hematopoietic tissue cells. The astakines and their vertebrate homologues, prokineticins, share similar functions in hematopoiesis; thus, studies of astakine-induced hematopoiesis in crustaceans may not only advance our understanding on the regulation of

invertebrate hematopoiesis but may also provide new evolutionary perspectives about this process.

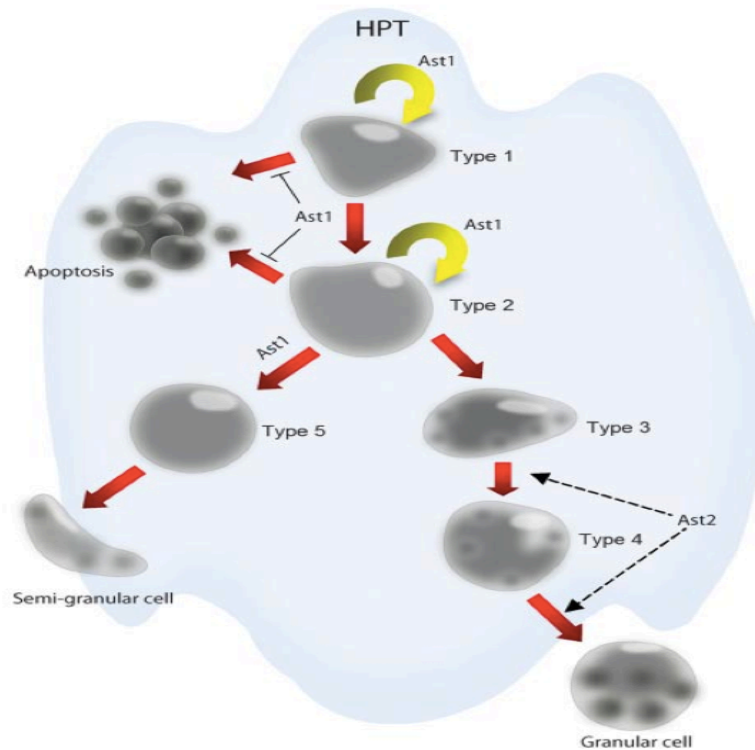


Figure 2.4. The effects of astakine on hematopoietic processes in crayfish. Astakine (Ast)1 promotes the proliferation of HPT cells and blocks apoptosis of these cells. Furthermore ast1 promotes the differentiation of HPT cells along the SGC lineage. Ast2 plays an important role in the maturation of the GC lineage. (Lin and Söderhäll, 2011)

2.3.3 Phagocytosis and apoptosis

Phagocytosis is a process carried out by phagocytes, which are hemocytes in crustaceans. The hemocytes have the capacity to recognize and ingest non-self molecules, such as bacteria, spores or senescent cells of their own. In crustaceans, phagocytes can be found free in the hemocoel, on the surface of arterioles of the hepatopancreas or in the gills. Phagocytes eliminate both Gram-negative and Gram-positive bacteria in crustaceans. Moreover, some cellular or cell-free hemolymph factors help phagocytosis (Tyson and Jenkin, 1974). This kind of opsonic effect of plasma lectins has been found in shrimps. Encapsulation is a multi-cellular response to eliminate foreign particles that cannot be destroyed by

humoral mechanism. This process kills pathogens or restricts their movement and growth.

Apoptosis is a genetically programmed cellular suicide process that eliminates unwanted or diseased cells. It plays important roles in embryogenesis, homeostasis, insect metamorphosis, and immunity (Opferman and Korsmeyer, 2003). Caspases, a family of structurally related cysteine proteases, play a vital role at various stages of the apoptotic process for being the main proteins in the initiation and execution stages of apoptosis (Kurokawa and Kornbluth, 2009). The silencing of the caspase gene of *Marsupenaeus japonicus* (PjCaspase) resulted in the increase of virus copies, above data indicating that apoptosis played a key role in antiviral process of shrimp (L. Wang et al., 2008). The enhancement of the apoptotic activity can effectively inhibit WSSV infection in shrimp (Zhi et al., 2011) resulting in decreases mortalities of WSSV-infected shrimp. On the other hand, viral miRNA can inhibit caspase 8 expression in *Marsupenaeus japonicus*, subsequently repressing the hemocytes apoptosis (T. Huang et al., 2013). It suggested that the caspase plays the key role in virus-host interaction by modulating apoptosis activity.

2.4 Antimicrobial peptides (AMPs)

AMPs as products of the immune response play important roles in killing or cleaning the invading pathogens directly (Bachère et al., 2000). In *Drosophila*, their production is regulated by the Toll and IMD pathways (De Gregorio et al., 2002). Even though the control mechanism of AMPs has not been clearly revealed, two important immune cascades, proPO and the clotting cascade have been proven to be closely related to the AMP regulation (Amparyup et al., 2013) and critical for the immune defense of crustaceans. Until now, several different types of AMPs have been identified in shrimps.

Penaeidins: The penaeidins are a family of antimicrobial peptides that appear to be expressed in all penaeid shrimps (Gueguen et al., 2006). They are mainly constitutively expressed in hemocytes (Kang et al., 2004). Penaeidins are responsive to pathogens and may provide surface protection to prevent or combat infection. Dramatic changes in penaeidin expression were observed in circulating hemocytes due to response to microbial challenge (Bachère et al.,

2000; Destoumieux-Garzon et al., 2001). Other research indicates that hemocytes, that contain penaeidins in granules, release their contents extracellularly and may lyse in the process of delivering penaeidins to the site of infection (Muñoz et al., 2002). Thus, penaeidins probably are involved in coating microbes for immune cell (phagocyte) recognition in addition to their antimicrobial activities. Much of this work has been corroborated (verified) independently in different species with more evidence being put forth of novel penaeidin activities such as agglutination of bacteria (Munoz et al., 2004).

Crustins: About 50 crustin-like genes have been reported in 20 different crustaceans species (Smith et al., 2008). Crustins are small peptides (~12 kDa) with a characteristic carboxy-terminal whey acidic protein (WAP) domain. Two main types of crustins have been proposed. Type 1 crustins, which phylogenetically group as part of the Pleocyemata and have a cysteine rich region positioned upstream of the WAP domain and Type 2 crustins from shrimp that have an additional glycine rich domain and cysteine rich region (Smith et al., 2008). Isoform variants are also recognized within this class structure (Brockton et al., 2007; Vargas-Albores et al., 2004). All full-length crustins have a signal sequence at the amino terminus indicating that, once produced within the granular hemocytes, they are secreted into the plasma where they appear constitutively. Crustins have been reported to have a role in the immune response to infection with Gram-positive and Gram-negative bacteria, fungi and viruses although many of these reports have only been based on studies of transcriptional regulation. Shockey et al. (2009) have also recently suggested that antimicrobial activity might be an indirect effect and that crustins may serve as opsonic factors like the penaeidins.

Antilipopolysaccharide factor (ALF): a small basic protein, was initially isolated and characterized in amebocytes of the horseshoe crab *Limulus polyphenus* (Tanaka et al., 1982). It binds to lipopolysaccharide and has strong antibacterial activity against, in particular, Gram-negative bacteria but also activity against Gram positive bacteria and fungi (La Vega et al., 2008). It has also been reported from RNA inhibition (RNAi) studies of ALF transcription in *Pacifastacus leniusculus*, that ALFs may directly interfere with viral replication (Liu et al., 2006).

3. CONCLUSIONS

The immune system of shrimp is a highly complex network of cells and immune factors produced by these cells. More focused studies on the interaction between pathogens and shrimp are necessary to understand the mechanism of the immune response against infections. Further work on gene function needs to be performed and some gene products would be possible to be used in aquaculture. Compared to the understanding on humoral immune response of shrimp, knowledge on cellular immune response of shrimp is still limited. Most of the work focused on the isolation of cDNAs of important genes and their functions were deduced by expression analysis at the molecular level. More work need to be developed at cell and tissue level. Although this work is very elementary, it can help to understand the immune response of crustaceans/shrimps to pathogen or stress. Phagocytosis and apoptosis were also considered as playing crucial roles in crustacean/shrimp response to microbial infection. Once more knowledge can be gained about the immune system of crustaceans/shrimps, the strategies for shrimp disease control can be developed.

Part 2: HEAT SHOCK PROTEINS AND ITS IMMUNE FUNCTIONS

1. WHAT ARE HSPS?

Heat shock response was firstly discovered by Ritossa when exposure of the *Drosophila* larval salivary gland to elevated temperature was observed to generate puffs in the polytene chromosome (Ritossa, 1962). In 1974, these puffs were proven to represent transcriptional induction of genes encoding heat shock proteins (HSPs) (Tissi res et al., 1974). It took even longer before it was recognized that they occurred in mammals and indeed at all evolutionary levels (Lindquist and Craig, 1988). In 1993, Welch (1993) referred HSPs as extrinsic chaperones, which are a suite of highly conserved proteins, of varying molecular weight (c. 16–100 kDa) produced in all cellular organisms when they are exposed to cellular stress. Although the response in the fruit fly was originally found to be up-regulated by exposure of their cells to heat, it is now recognized that the response is universal to all cells and other stressors such as anoxia ischaemia, toxins, protein degradation, hypoxia, acidosis and microbial infection will also lead to their up-regulation (Welch, 1993).

Intracellular counterparts of HSPs, referred to as chaperones, constitutive chaperones or heat shock cognates (Hscs), are also found within the cytoplasm of normal unstressed cells, representing 5–10% of the total protein in healthy growing cells (Pockley, 2003). Hscs are recognized by primarily residing in the cytosol, nucleus and mitochondria. They are universal to all cells and essential for various homoeostatic functions, including maintenance of protein structure and folding, supporting and repairing damaged cytoskeleton elements, assisting in the production and folding of intracellular proteins, enzymes and hormone receptors and maintenance of mitochondria, nuclear and cell wall lipoprotein membranes (Beckmann et al., 1990).

HSPs are categorized into several families and named according to their function, sequence homology and molecular mass in kilo-Daltons (kDa). The families primarily include HSP100, HSP90, HSP70, HSP60, HSP40 and several smaller HSP groups (**Table 2.3**). Many members of the HSP families have

counterparts, referred to as heat shock cognates (Hscs) that are expressed within the cell under normal non-stress conditions. They play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families such as HSP90 and HSP70 are critical to the folding and assembly of other cellular proteins (Gething and Sambrook, 1992). They and other molecular chaperones are also involved in regulation of kinetic partitioning between folding, translocation and aggregation as well as having a wider role in relation to the immune, apoptotic and inflammatory processes (Moseley, 2000; Pockley, 2003; Srivastava, 2002).

Table 2.3 The main families of heat-shock proteins (modified from Lindquist 1992; Pockley 2003)

HSP family	Members	Monomer mass (kDa)	Cell localization	Function
HSP100	ClpA ClpB ClpC HSP104	80–110	Cytoplasm, nucleolus, nucleus, chloroplast	Thermotolerance, ethanol tolerance, long-term spore viability
HSP90	HSP82 Grp94 HtpG	82–96	Cytoplasm, nucleus	Essential for viability; increased concentration required for growth at high temperatures
HSP70	DnaK grp78 hsc70, BiP Kar2, ssa, ssb, ssc, ssd HSC70(HSP72)	67–76	Cytoplasm, nucleus, mitochondria, chloroplasts, endoplasmic reticulum	Chaperone required for protein assembly, secretion, protein import into the endoplasmic reticulum and organelles; growth at high temperature
HSP60	GroEL, HSP65,	58–65	Mitochondria,	Chaperonin, assembly of

	cpn60, Rubisco- binding protein		chloroplasts	oligomeric proteins and folding of monomeric proteins; high concentration required for growth at elevated temperature
sHSPs	Many	18–40	Cytoplasm, nucleus	Protection from stress, apoptosis inhibition

The induction of HSP results from the binding of activated heat shock transcription factor (HSF) to key promoter regions, called the heat shock elements (HSE), in front of the heat shock genes. The major heat shock genes do not contain introns and so the messenger RNA can be immediately translated into new proteins within minutes of exposure to the stressor. In vertebrates like humans, mice, chicken and finfish (and also in plants), four HSFs have been identified. Of these, HSF1, HSF2 and HSF4 are ubiquitously expressed whereas HSF3 appears to be avian specific (Morimoto, 1998). In the yeasts and invertebrate *Drosophila*, only one HSF has been reported (Clos et al., 1990). The main heat shock factor responsible for regulating the heat shock genes in response to physiological and environmental stress is HSF1 (Voellmy, 1994) whereas activity of HSF2 is more selective, and is mostly induced during differentiation and early development (Pirkkala et al., 2001). Stress does not change the amount of HSF1 (Rabindran et al., 1991), but changes the inactive form of HSF1 to a transcriptionally active state that results in the expression of HSPs.

Usually, HSF1 is present in the cytoplasm as a latent monomeric molecule that is unable to bind to DNA. Under stressful conditions, HSF1 is hyperphosphorylated in a ras-dependent manner by members of the mitogen-activated protein kinase sub-families and converted to induce phosphorylated trimers that have the capacity to bind DNA (Knauf *et al.*, 1996; Kim *et al.*, 1997). The phosphorylated trimers translocate from the cytoplasm to the nucleus and bind to the promoter regions of the heat shock genes (Jurvich *et al.*, 1992; Bruce *et al.*, 1993). Upon binding of HSF1 to its target, HSP70 is expressed through

normal transcription and translation processes (**Fig.2.5**). HSF2 has the characteristics of a temperature-sensitive protein; it is inactivated when exposed to raising temperature, and sequestered to the cytoplasm, and is thereby prevented from interference with HSF1 activity in stressed cells (Mathew et al., 2001).

The induction of stress proteins in response to stress is only transient, as a prolonged and inappropriate presence of protein-binding molecules would adversely affect protein homeostasis and a variety of intracellular functions, leading to inappropriate growth control and possibly cell death (Todryk et al., 2003). One mechanism that regulates HSP70 expression is the binding of HSP70 to the transactivation domain of HSF1, leading to the release of the HSF1 bound to HSE of the DNA, and subsequent dissociation of the trimeric form back to the inactive monomers that move back into the cytoplasm (Abravaya *et al.*, 1991). A second mechanism regulating HSP synthesis is the interaction between heat shock protein binding factor 1 (HSBP1), the active trimeric form of HSF1, and HSP, resulting in inhibition of the capacity of HSF1 to bind to DNA (Satyal *et al.*, 1998). HSBP1 is mainly localized in the nucleus, and HSBP1 mRNA is present at high concentrations in various cell lines and animal tissues that are unaffected by heat shock (Satyal et al., 1998).

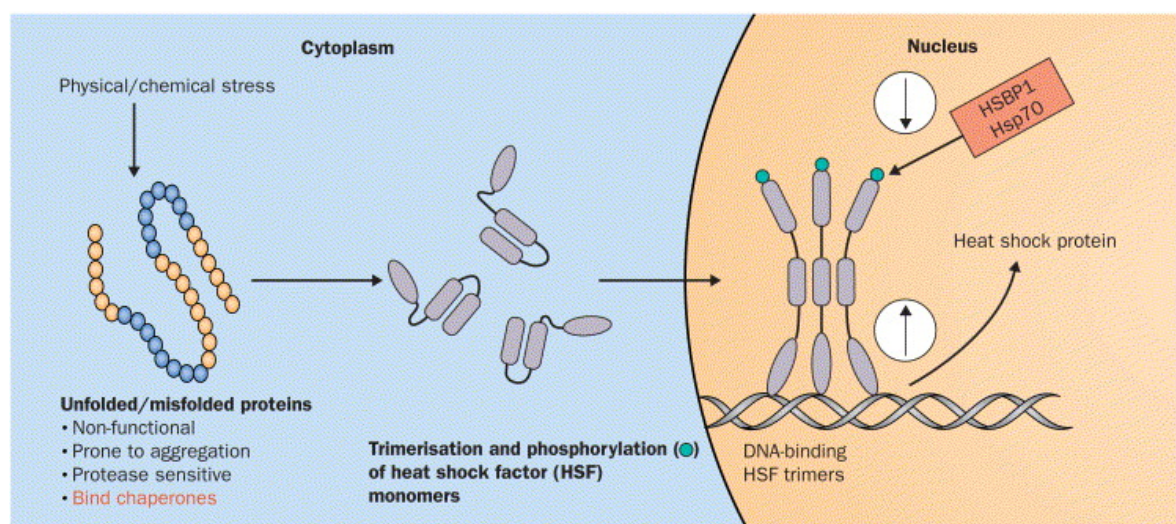


Figure 2.5 Induction and regulation of heat shock protein expression. Physical or chemical stress induces production of unfolded or misfolded proteins. Heat shock factor monomers in the cytoplasm form trimers, are phosphorylated, and translocate into the nucleus. HSF homotrimers bind to heat shock protein gene promoter regions,

leading to induction of HSP gene transcription. HSP70 gene transcription is downregulated by interaction of HSP70 or HSBP1 with the HSF trimers. (Pockley 2003)

2. HSPS AND THE IMMUNE RESPONSE

While originally it was considered that the HSPs response to cellular stressors was a short-term functional response, with a range of essentially housekeeping and cytoprotective functions (Pockley, 2003), it is now becoming clear that the HSP response also plays a significant role in regulation of the mammalian immune response in both the innate and adaptive immunity (Feder and Hofmann, 1999; Moseley, 2000). Numerous studies have implicated HSPs in various aspects of the immunological response to antigens, leading to the proposal that these proteins carry out a “moonlighting” function as “chaperokines” (Asea, 2006; Henderson et al., 2010; Javid et al., 2007) . These studies have shown that HSPs act both as adjuvants by triggering Toll like receptors (TLRs) on cells of the innate immune system, in particular macrophages and Dendritic cells (DCs), and also as carriers of antigens by providing a mechanism of chaperoning polypeptides for loading in Major histocompatibility complex (MHC) molecules and the subsequent facilitation of induction of acquired immunity (**Fig. 2.6**).

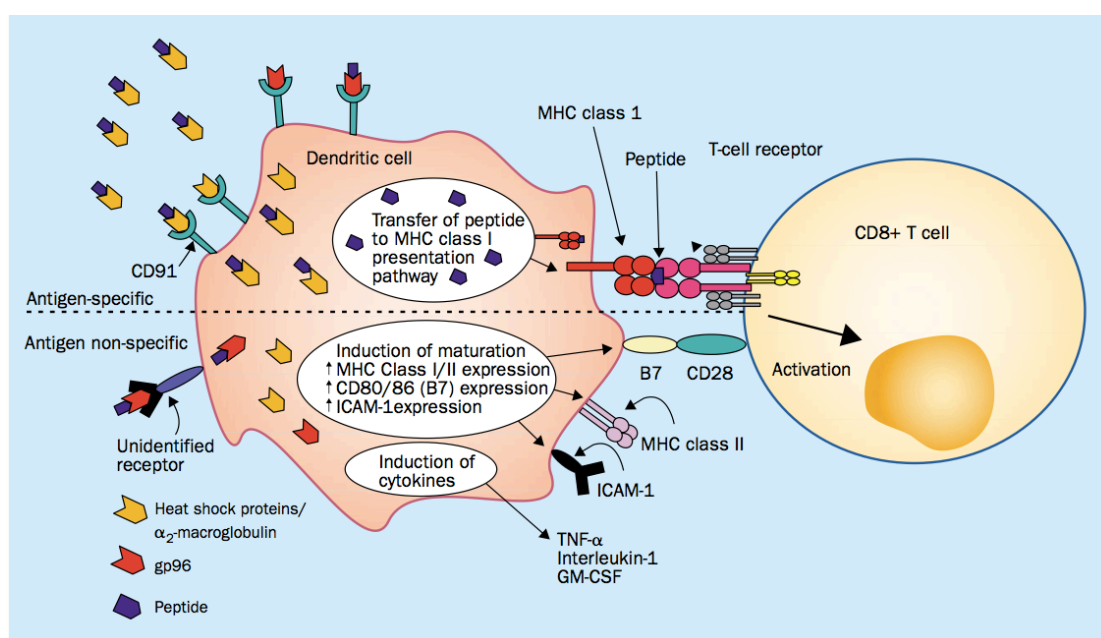


Figure 2.6: Effects of heat shock proteins on dendritic cells, and induction of peptide-specific immunity GM-CSF=granulocyte-macrophage colony stimulating factor. The heat shock proteins calreticulin, Hsp70, Hsp90, and gp96, and the serum protein α 2-macroglobulin (either complexed with peptide or uncomplexed) are internalised by dendritic cells through the CD91 molecule (α 2-macroglobulin receptor), and/or an as yet unidentified receptor in the case of gp96. Chaperoned peptides are delivered into the MHC class I presentation pathway for subsequent presentation to MHC class I-restricted CD8⁺ T cells. Hsp70, Hsp90 and gp96 have been shown to concomitantly induce maturation of dendritic cells, as shown by the induction of MHC class II, B7 and ICAM-1 molecule expression, and their release of cytokines. (Pockley, 2003)

2.1 Innate immunity (mammalian)

Most studies about the role of HSPs in innate immunity have been conducted with mammalian cell lines. Initially HSPs were thought to be exclusively intracellular proteins that were only released into the cellular environment upon cellular injury or necrosis, but not apoptosis and, as such, they were not generally regarded as pathogen associated molecule patterns (PAMPs) but considered to be “danger associated molecular patterns” (DAMPs) (Basu et al., 2000). DAMPs are molecules that serve as alternative ligands for PRRs but signal the presence of cellular damage, as distinct from the presence of pathogens, thus also activating the innate immune response (Bianchi, 2007). However it is now apparent that HSPs can be actively secreted into the extracellular environment by tumor cells or released from cells undergoing necrotic lysis in response to cytotoxic lymphocyte (CTL) or natural killer (NK) action, or viral infections (Feng et al., 2003; Mambula and Calderwood, 2006; Merendino et al., 2010).

Mycobacterial HSP70 can stimulate cytokine production in monocytes, by interacting with both TLR2 and 4, in a CD14-dependent manner (Lehner et al., 2000). This ability to activate innate immunity was localized to the C-terminal peptide binding region (aa359–610) of HSP70 which elicited production of IL-12, TNF- α , RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), and nitric oxide (NO) in THP-1 cells, whereas the N terminal nucleotide binding region of HSP70 (aa 1–358) did not (Lehner et al., 2004; Y.

Wang et al., 2002). The full mycobacterial HSP70 molecule appears also to contain epitopes that inhibit DCs maturation and promote anti-inflammatory cytokines (IL- 10) (Y. Wang et al., 2005). Further studies revealed that only mycobacterial HSP70 but not human HSP70 induced this observation, with bacterial HSP70 (DnaK) and human HSP70 appearing to bind to different regions of CD40 on macrophages and DCs (Becker, 2002; Y. Wang et al., 2001). It has also been reported that mycobacterial HSP70 binds to CCR5 and CD40 on human DCs, stimulating production of IL-12p40 and TNF- α (Whittall et al., 2006). Thus it appears that HSP70's ability to stimulate cells contributing to the innate immune system is dependent on the source of HSP, as mammalian and microbial forms appear to use different receptors (Becker, 2002; Bendz et al., 2008; Lehner et al., 2004; Y. Wang et al., 2005; 2002; Whittall et al., 2006). Moreover, it appears that while mycobacterial HSP70 stimulates an innate immune response, the situation with mammalian HSP70 is more variable and some members of this gene family may down regulate the immune response instead (Henderson, 2010; Javid et al., 2007; Panayi and Corrigan, 2006; Y. Wang et al., 2005). This has led to the proposal that these HSPs may have a distinct role as "resolution associated molecular patterns" (RAMPs) that lead to the resolution of inflammation induced by activation of the innate immune response by DAMPs and PAMPs (Panayi and Corrigan, 2006; Shields et al., 2011).

Although many studies using recombinant proteins that have been generated in *E. coli* have failed to recognize that contamination with PAMPs is a potential problem, those working on the cell signaling actions of molecular chaperones have recognized that microbial contaminants might be a problem, and steps have been taken to avoid such contamination (Henderson, 2010). A series of problems have been raised by various groups of researchers. Gao et al (2002) worked on the problems related to lipopolysaccharide (LPS) contamination of HSP60 and HSP70 recombinant proteins. Ye and Gan (2007) suggested that flagellin contamination of HSP70 was responsible for its biological actions. The flagellin contamination of HSP70 is countered by the work of Figueiredo and co-workers who have expressed HSP70 in mammalian cells and have generated LPS-free protein that still stimulates T lymphocytes (Figueiredo et al., 2009). A final contaminant responsible for the cellular activation of HSP70 proteins is,

apparently, nucleotides (Bendz et al., 2008). The possibility that the leukocyte-stimulating activity because of: (1) the recombinant HSP70 protein is subject to further chromatography which would remove nucleotides and (2) the C-terminal fragment of *M. tuberculosis* HSP70, purified on a nickel affinity column, with no involvement of nucleotides, was biologically active (Y. Wang et al., 2001). Thus, it is unlikely that the immune stimulating activity of *M. tuberculosis* Hsp70 is due to nucleotides contaminated in protein preparation.

2.2 Adaptive immunity and cross-presentation of HSPs

The most interesting feature of the uptake of HSP-chaperoned peptides by antigen present cells (APCs) is their availability for cross-presentation, which is the ability of exogenous antigens to enter the endogenous loading pathway of the major histocompatibility complex (MHC) Class I molecules and thus prime CD8+ T cells (Ichiyanagi et al., 2010; Murshid et al., 2012). Cross-presentation can occur *via* one of two pathways, either the vacuolar/endocytic pathway (nonclassical MHC I loading) or the cytosolic pathway (classical MHC I loading) (Murshid et al., 2012; Tobian et al., 2004). In the vacuolar/endocytic pathway, antigen is taken up by the cell by phagocytosis, and formation of phagolysosomes provides the appropriate environment for the production of peptide fragments that are then loaded onto MHC I molecules within this compartment: the source of MHC I molecules is believed to be from membrane recycling or from ER-phagosome fusion (Murshid et al., 2012; Tobian et al., 2004). In the cytosolic pathway, antigen is once again taken up by the cell *via* phagocytosis and, once internalized, the antigen is trafficked to the cellular cytosol (through the transmembrane protein Sec61) and enters by the classical MHC I pathway of loading and this translocation to the cytosol requires HSP90 assistance (Ichiyanagi et al., 2010; Murshid et al., 2012).

2.3 HSPs as danger signals to the immune system

Until recently, the immune system was seen solely as a defense system with its primary task being the elimination of unwanted microbial invaders. Currently, however, the functional significance of the immune system has obtained a much wider perspective, to include among others the maintenance and restoration of

homeostasis following tissue damage. In this latter aspect, there is a growing interest in the identification of molecules involved, such as the so-called danger or damage-associated molecular patterns (DAMPs), also called alarmins. Since heat shock proteins are archetypical molecules produced under stressful conditions, such as tissue damage or inflammation, they are frequently mentioned as prime examples of DAMPs. See for instance also the recent review of Chen and Nuñez (2010). Contrary to this description, Broere et al. (2011) recently presented some of the arguments against a role of heat shock protein as DAMPs: in a case of bacteria-host symbiosis, HSPs exhibit a dampening effect on immune activation and have the capacity to promote immune homeostasis. Thus, the immune functions of HSPs need to be carefully investigated under different circumstances.

3. HSPS AND INFECTIOUS DISEASES IN FISH AND SHELLFISH

It is well recognized that a non-lethal heat shock will activate the transcription and synthesis of HSPs protecting animals against subsequent stressors. In addition there is less information on the effect of a heat shock or other significant stressor on subsequent resistance to pathogenic stressors and thus the role of HSPs in disease pathogenesis (Dalmo, Ingebrigtsen & Bøgwald 1997). HSPs chaperone intracellular peptides are released from dead or damaged cells following infection. Apart from triggering a concomitant inflammatory stimulus they deliver these peptides as complexes to antigen presenting cells for activation of T-lymphocytes. This is a particularly significant process, very different from the response to apoptotic cell death and it facilitates the subsequent host response to the pathogens responsible for the necrosis (Pockley 2003).

The first study on the significance of HSPs activity in the host response to fish or shellfish pathogens was by Forsyth et al. (1997) who infected coho salmon, *Oncorhynchus kisutch* (Walbaum), with *Renibacterium salmoninarum* and followed the levels of HSP70 in kidney and liver over a period of 63 days post-infection. HSP70 levels were significantly increased over those of mock injected controls and they pointed out that such elevated levels, if induced by disease in wild populations, would present problems for any environmental surveys based

on HSP levels as indicators of environmental stress.

In a subsequent study on *Vibrio anguillarum* infection in rainbow trout, Ackerman & Iwama (2001) followed the course of infection over an 8-day period and showed that there was a progressive increase in circulating bacteria numbers and plasma cortisol levels, but the cortisol levels peaked before any clinical signs were observed. The peak in liver HSP70 levels correlated with the highest cortisol levels but HSP70 levels in the kidney only reached those high levels 24 h later. Deane & Woo (2005) extended the findings of Ackerman & Iwama (2001) in their study of HSP activity and cytoprotection from vibriosis in the silver sea bream *Sparus sarba*. Immunoassays of different HSP families following infection demonstrated that the expression of HSP90 and HSP60 remained unchanged throughout the disease process, but expression of the HSP70 family decreased in kidney and liver tissues from an early stage of the infection. Reverse transcriptase polymerase chain reaction analysis of the HSP70 transcription-inducing factor, HSF1, demonstrated that down-regulation of this transcription induction factor mediated the loss of cytoprotective function during the course of infection.

HSP activity in relation to pathogenesis in crustaceans has been less well studied, with interest centered mainly on other aspects of HSP function such as the response to traumatic injury (Xue & Grossfeld, 1993) or the diapause process in the brine shrimp, *Artemia franciscana* (MacRae 2003), and their role in the heat resistance of vent dwelling shrimp (Ravaux et. al. 2003). Recent experimental studies by Sung and his colleagues using a gnotobiotic brine shrimp-*Vibrio* model have, however, contributed significantly understanding of the significance of HSPs in crustacean pathology (Sung et al., 2007,2009). They demonstrated that a non-lethal heat shock of 37 °C for 30 min increased expression of HSP70 in the shrimp. If the shrimp were then given a 6-h recovery period, a subsequent challenge with *Vibrio campbellii* or *Vibrio proteolyticus* gave a 100% increase in larval survival over un-shocked controls (Sung et al., 2007), but hypothermic shock or acute osmotic insult failed to produce HSP70 chaperones and did not protect against infection (Yeong Yik Sung et al., 2008). In a study of effects of white spot syndrome virus (WSSV) infection on gene expression in *Penaeus monodon*, Huang et al. (2008) showed that HSP21 gene

was highly expressed in all organs of the shrimp but down-regulated with WSSV infection, suggesting that regulation of this gene and its product were seriously inhibited by the infection. Such down-regulation of HSP genes in association with infection was also reported by Deane & Woo (2005) in sea bream and may possibly represent a significant element in the pathogenesis of infections in both fish and crustaceans.

4. CONCLUSION

Understanding the role and significance of the HSP/chaperone system in farmed aquatic animals is as yet limited. However rapid advances are being made in medical and veterinary research, in terms of fundamental understanding of the HSP genes, their regulation and the effects of their products on both the normal housekeeping of the cell and on cell signalling, inflammation and the immune response. The recent application of that understanding to a variety of human and veterinary clinical situations and the first developments of pathogen-derived HSP vaccines, such as bacterial HSPs, a recombinant complex of HSP and viral capsid protein, provides an indication of their potential value in many areas of aquatic sciences.

CHAPTER 3

BACTERIAL HSP70 (DnaK) IS AN EFFICIENT IMMUNE STIMULATOR IN *LITOPENAEUS VANNAMEI*[†]

[†] Adapted from:

Hu, B., Phuoc, L.H., Sorgeloos, P., Bossier, P., 2014. Bacterial HSP70 (DnaK) is an efficient immune stimulator in *Litopenaeus vannamei*. Aquaculture 418-419, 87-93.

Abstract

There is increasing evidence suggesting that heat shock proteins (HSPs), especially the conserved HSP70, are able to activate the immune response. In our research, we aimed at verifying the role of DnaK (the bacterial HSP70 homologue) as a immune activator in specific-pathogen-free (SPF) *Litopenaeus vannamei* either by injecting them with recombinant full length DnaK or a chemically synthetic 50 amino acid fragment peptide which is part of the peptide binding domain of DnaK (DnaK₄₄₂₋₄₉₁). The expressions of two types of immune-related genes (transglutaminases and prophenoloxidasases) were monitored *via* quantitative real time PCR (qRT-PCR). The results showed that DnaK and DnaK₄₄₂₋₄₉₁ are able to significantly up-regulate transglutaminase-1 (TGase-1) and prophenoloxidase-2 (proPO-2) expression within 12 hours post injection. The synthetic peptide DnaK₄₄₂₋₄₉₁ is free of lipopolysaccharide (LPS) or any other bacterial components potentially contaminating a recombinant DnaK preparation. Hence the data suggested that DnaK, as such, stimulates the immune system of *L. vannamei* in a specific way (proPO-2, TGase-1). These findings indicate that DnaK is an efficient immune stimulator in *L. vannamei* immunity.

1. Introduction

Litopenaeus vannamei is one of the most important aquaculture species in the world. Like other crustaceans, it does not have an acquired immunity; instead they have an innate immune system or so-called non-specific immunity. This kind of immunity encompasses different reactions, e.g. melanization by activating the prophenoloxidase-activating system (proPO-AS), hemolymph coagulation, phagocytosis, encapsulation of foreign particles (Söderhäll and Cerenius, 1998). In crustaceans, transglutaminase (TGase) is an active enzyme secreted by hemocytes circulating in the hemolymph. It rapidly initiates polymerization of clotting proteins entrapping foreign particles and stopping bleeding when hosts are traumatized (Cerenius and Söderhäll, 2011; Loof et al., 2011). TGase-silenced shrimps were found highly susceptible to microbial infection while a higher bacterial count was observed in their hemolymph (Fagutao et al., 2012; Maningas et al., 2008; Söderhäll and Cerenius, 1998). Hemocytes are also the source of prophenoloxidase, which is the zymogen of phenoloxidase (PO). The proPO-AS is activated by several factors and leads to conversion of proPO to PO. The activated PO eventually catalyzes the oxidation of tyrosine-derived phenols to quinones believed to be directly toxic to microorganisms, and to polymerize non-enzymatically to form insoluble melanin that physically encapsulates pathogens (Cerenius and Söderhäll, 2011; 2004; Loof et al., 2011). Both TGase and proPO are vital components of the crustacean innate cellular and humoral immune defense.

Heat shock proteins (HSPs) are abundant and ubiquitous in both eukaryotes and prokaryotes, performing a multitude of housekeeping and cytoprotective functions (Srivastava, 2002). They are categorized into several families based on their molecular weight, such as HSP60, HSP70, and HSP90 etc. Among those HSPs families, HSP70 is the best studied and most highly conserved (Sanders, 1993). Increasing evidence suggests that HSP70 plays an important role in regulation of the early innate immune response. In higher eukaryotes, HSP70 stimulates innate immune cells e.g. dendritic cells, macrophages, monocytes, producing several pro-inflammatory cytokines via certain receptors and a signal transduction pathway (Wallin et al., 2002). In crustaceans, feeding either heterologous or homologous HSP70 has been found to successfully improve the

PO activity and protect *Artemia* from *Vibrio* infection (Baruah et al., 2010). There is, however, still an ongoing debate about the immune-stimulatory nature of recombinant HSP70, as recombinant HSP70 processed from Gram-negative bacteria like *E.coli* is potentially contaminated with LPS, flagellin and nucleotides which can activate the immune system in a very similar way (Bendz et al., 2008; Todryk et al., 2003; Wallin et al., 2002; Ye and Gan, 2007).

In our study, we aimed at verifying whether the HSP70 homologue DnaK is responsible for inducing an immune response in *L. vannamei*. To achieve this, full length bacterial HSP70 (DnaK) was purified from recombinant *E.coli* and injected into specific-pathogen-free (SPF) shrimps. The expression of two immune-related genes, prophenoloxidase (proPO-1, proPO-2) and transglutaminase (TGase-1, TGase-2) was monitored within 12 h post injection. In addition, a chemically synthetic DnaK epitopic peptide (DnaK₄₄₂₋₄₉₁) was tested in the same way, as microbial contaminant-free control. The results showed that the expression files of two significantly responding genes (proPO-2 and TGase-1) were very similar, suggesting that DnaK as such does stimulate the expression of genes considered playing a central role in the immune defense of *L.vannamei*.

2. Materials and methods

2.1 Production of recombinant DnaK

The *E.coli* strain E_{native} (overexpressing DnaK with a hexahistidine-tag) was constructed and described previously (Baruah et al., 2013). These bacteria were stored in 40% glycerol at -80°C, then grown at 37°C for 24 h on Luria-Bertani (LB) agar containing kanamycin, and finally on a rotary shaker to log phase (OD₆₀₀ 0.4-0.6) in LB broth. Overproduction of DnaK was induced by adding L-arabinose (0.5 mg ml⁻¹) at 37°C for 4 hours. After induction, the bacteria were transferred into sterile tubes, centrifuged at 2200g for 15 min, suspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen). Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in a mini beads beater (Biospec, USA) and subsequently centrifuged at 2200 g for 1 min at 4 °C. The supernatant protein was taken for DnaK isolation.

For DnaK isolation, Dynabeads® (Dynabeads® His-Tag Isolation & Pull down, Invitrogen) were used according to the recommended protocol. Briefly, cell lysate was incubated with Dynabeads® for 10 minutes. The Dynabeads® were washed with fresh DPBS 4 times. Finally the DnaK was eluted into elution buffer from the Dynabeads®. The elution buffer was exchanged to DPBS using the Amicon® Ultra Centrifugal Filter (Millipore) according to the manual. The isolated DnaK was stored at -80°C as aliquots for future application.

2.2 Recombinant DnaK analysis

The isolated recombinant DnaK concentration was determined by the Bradford assay (Bio-rad). A 1 µg DnaK sample was combined with loading buffer, vortexed, heated at 95°C for 5 min and electrophoresed in 10% SDS-PAGE gels. Gels were either stained with Bio-safe Coomassie stain (BioRad) or transferred to polyvinylidene fluoride membranes (BioRad) for antibody probing. Membranes were incubated with blocking buffer, 50 ml of 1x PBS containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin (Sigma), for 60 min at room temperature and then with monoclonal antibody 8E2/2, raised in mouse to DnaK, at the recommended dilution of 1:1000 (Stressgen Bioreagents) as primary antibody. Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO, USA). Detection was done with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) in association with 0.01% (v/v) H₂O₂ in 0.1 M Tris HCl (pH 7.6). Both SDS-PAGE and Western Blot showed a highly purified single band (see appendix I).

The endotoxin lipopolysaccharide (LPS) content of the recombinant DnaK was measured with a chromogenic endotoxin detection kit (GenScript, NewJersey, USA). The endotoxin content of the recombinant DnaK stock was 0.45 EU/mg DnaK.

2.3 DnaK fragment peptide (DnaK₄₄₂₋₄₉₁) synthesis

A 50 amino acid peptide derived from the peptide-binding domain of DnaK, named DnaK₄₄₂₋₄₉₁ (QGE RKR AAD NKS LGQ FNL DGI NPA PRG MPQ IEV TFD IDA DGI LHV SAK DK) was custom-made by Eurogentec (Belgium). The stock

solution of DnaK₄₄₂₋₄₉₁ was made by dissolving DnaK₄₄₂₋₄₉₁ powder in water (Gibco®, Invitrogen) to a final concentration of 10 µg/µl and kept at -80°C as aliquots. Before injection, the DnaK₄₄₂₋₄₉₁ stocking solution was diluted with Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen).

2.4 Experimental animals and conditions

Specific-pathogen-free (SPF) *L. vannamei* were imported from Sy-Aqua Siam Co. Ltd., (Thailand). Animals were certified to be free of Taura Syndrome Virus (TSV), WSSV, Yellow Head Virus (YHV) and IHHNV by the Thai Department of Fisheries. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL8–12). They were kept in a recirculation system at a water temperature of 28–29 °C, 34 g l⁻¹ salinity and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high-performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Hemolymph of *L. vannamei* was examined randomly by plating on Marine agar and no bacterial growth was observed. Shrimps for experimental purpose (MBW = 15.6 ± 1.3 g) were housed individually in covered 10 l aquaria, filled with Instant Ocean® seawater prepared with distilled water at a salinity of 35 g l⁻¹, provided with constant aeration and maintained at 27 ± 1°C by air heaters. Shrimps were acclimated under the latter conditions for 3 days before subsequent treatment. Feeding was skipped for 24 h prior to the injection.

2.5 Recombinant DnaK and Peptide DnaK₄₄₂₋₄₉₁ treatment

Two doses of recombinant DnaK protein were injected to shrimps, a high dose (5 µg) and a low dose (0.05 µg) per animal (the DnaK dosage calculation see appendix II). In a control experiment, two equivalent doses of DnaK₄₄₂₋₄₉₁ were applied, 0.3 µg and 0.003 µg based on their respective molecular weight. They were dissolved in 100 µl Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium and no magnesium, Gibco®, Invitrogen) and then intramuscularly injected on the tail. As blank, DPBS was injected. The hemolymph was collected

at 5 time points post injection, 1, 3, 6, 9 and 12 h post injection (hpi). Three animals from each treatment were sacrificed at every time point.

2.6 Total RNA isolation and cDNA synthesis

Hemolymph (200 µl) was collected from each shrimp using 1 ml syringe with 20G needle containing 300 µl of precooled anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4). After centrifugation of 250 g at 4°C for 5 minutes, the hemocyte pellets were quick frozen in liquid nitrogen and then preserved at -80°C for RNA extraction. RNA samples from different shrimps were processed and analyzed individually.

Total RNA was extracted from hemocytes using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Fermentas) to remove genomic DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 0.5 µg total RNA using the RevertAid™ 6H minus First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions.

2.7 Quantitative real-time PCR (qRT-PCR) analysis

Expression of two transglutaminase genes (TGase-1, TGase-2) and two prohenoloxidase genes (proPO-1, proPO-2) in *L. vannamei* were analyzed by qRT-PCR using a pair of specific primers designed on the basis of several *L. vannamei* cDNA sequences (**Table 3.1**, GeneBank accession no.). The β -actin was used as reference gene (the selection of reference gene see appendix III). Real-time PCR was performed in an ABI StepOne™ Real-time System thermal cycler (Applied Biosystem) in a total volume of 20 µl containing 10 µl of 2× SYBR Green qRT-PCR Master mix (Applied Biosystems), 0.5 µl (each) forward/reverse primers (10 µM), 5 µl of cDNA template and 4 µl nuclease-free water (Sigma). The thermal cycle parameters used for the real-time amplification were: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 30 s and elongation at 60°C for 30 s.

Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne™ software (v 2.2.2, Applied Biosystem) at the end of each elongation step.

Table 3.1 Gene bank accession No. and primer sequences used in qRT-PCR

Gene	Gene Bank Accession No.	Primers
Transglutaminase-1 (TGase-1)	EF081004.1	F: GAGCTTCAAGATCGAGGATCGA R: GCTGGTGTTCGTAGCGGTTATC
Transglutaminase-2 (TGase-2)	EU164849.1	F: CTCAGCGTGAACCTCGCCTTA R: CCTGCTCAGTGTACGGTCT
Prophenoloxidase-1 (proPO-1)	AY723296.1	F: AACTCCATTCCGTCCGTCTG R: GGCTTCGCTCTGGTTAGGAT
Prophenoloxidase-2 (proPO-2)	EU373096.1	F: CTCAGCGTGAACCTCGCCTTA R: CCTGCTCAGTGTACGGTCT
β-actin	AF300705.2	F: GATGTGTGACGACGAAGTAG R: GATACCTCGCTTGCTCTGG

2.8 qRT-PCR data analysis

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 0.5 µg of RNA isolated from hemocytes samples. Serial dilutions of cDNA were amplified by real-time PCR using gene specific primers. ΔC_T (average C_T value of target – average C_T value of β-actin) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 1 for all of the target genes. Therefore, the amplification efficiency of reference and the target genes is equal. The expression of the target genes was normalized to the endogenous control β-actin by calculating ΔC_T (Livak and Schmittgen, 2001):

$$\Delta C_T = C_{T, \text{target}} - C_{T, \beta\text{-actin}}$$

and expressed relative to blank group (calibrator) by calculating $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T, \text{calibrator}}$$

The relative expression was then calculated as

$$\text{Relative expression (fold)} = 2^{\Delta\Delta C_T}$$

2.9 Statistics

Data were analyzed by two-way analysis of variance (ANOVA) with post-hoc Bonferroni's correction (GraphPad Prism 5.0; GraphPad Software) to determine significance. All data were expressed as mean \pm SEM. Values were considered significantly different if $p < 0.05$.

3. Results

3.1 Transglutaminase (TGase) expression

To determine if the recombinant DnaK has an immune-stimulatory effect in *L. vannamei*, gene expression of the TGase was analyzed by qRT-PCR during a period of 12 hours post injection. Up to now, two TGase genes named TGase-1 and TGase-2 have been cloned and sequenced (**Table 3.1**). As shown in **Figure 3.1A**, within 12 h, the expression of TGase-1 in *L. vannamei* injected with either a high dose or low dose of DnaK is different relative to the blank. A high dose of DnaK exhibited a highly significant ($p < 0.001$) 10-fold increase in the relative abundance of TGase-1 mRNA transcript at 6 h post injection (6 hpi), after which it declined. In contrast, the low dose of DnaK up-regulated the TGase-1 expression 60-fold at 6 hpi ($p < 0.001$).

The same pattern of TGase-1 expression was obtained by injecting the low dose of DnaK₄₄₂₋₄₉₁ (**Figure 3.1B**). The gene expression of TGase-1 was 15-fold higher ($p < 0.001$) at 6 hpi. However, the high dose of DnaK₄₄₂₋₄₉₁ had no significant effect on TGase-1 gene expression.

On the other hand TGase-2 gene expression did not show any significant regulation during the 12 hours post injection (data not shown).

3.2 Prophenoloxidase (proPO) expression

The prophenoloxidase-activating system (proPO-AS), present in the open circulatory system of crustaceans and other invertebrates, is regarded as the

most important component of the innate immunity and plays a vital role in defense against pathogens. In *L.vannamei*, two proPO genes were identified (proPO-1 and proPO-2). Since proPO-1 did not show any significant regulation during 12 h post injection (data not shown), here, the immune stimulating action of recombinant DnaK and DnaK₄₄₂₋₄₉₁ were evaluated through proPO-2 (**Figure 3.2**). Upon injection of a high dose (5 µg) and a low dose (0.05 µg) of DnaK (**Figure 3.2A**), proPO-2 gene expression was up-regulated 5-fold and 4-fold respectively during the first 3 h ($p<0.001$). Hereafter, proPO-2 expression decreased.

The same analysis was done to determine the influence of DnaK₄₄₂₋₄₉₁ on the proPO-2 gene expression (**Figure 3.2B**). The high dose (0.3 µg) led to a significant increase in the transcript level of proPO-2 mRNA at 3 hpi (6-fold, $p<0.001$). In the following 9 hours the proPO-2 transcript level reduced over time. A similar expression pattern was observed upon the injection of a low dose (0.003 µg).

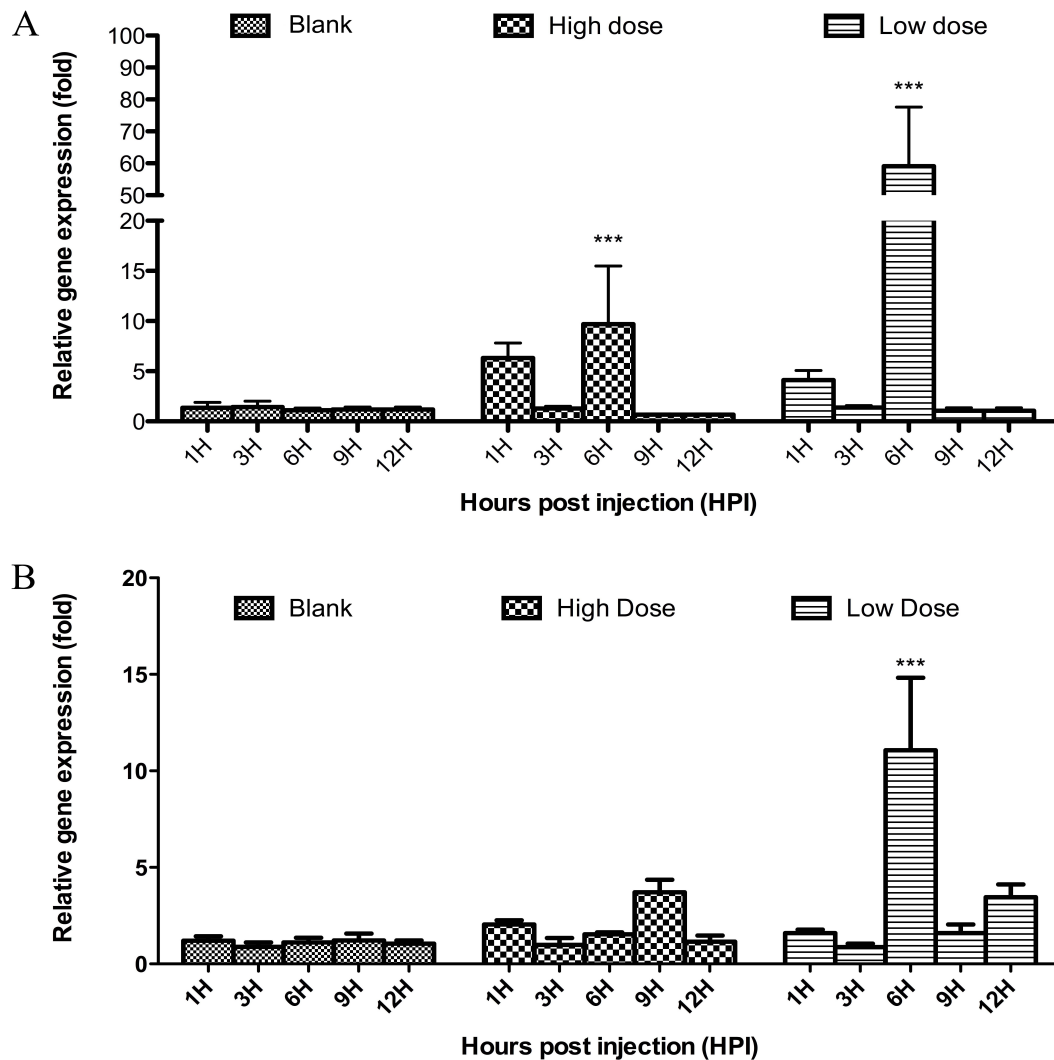


Figure 3.1 Expression of transglutaminase-1 (TGase-1) mRNA in *L. vannamei* injected with DnaK or the 50 amino acid peptide DnaK₄₄₂₋₄₉₁. *L. vannamei* were injected with either recombinant DnaK or DnaK₄₄₂₋₄₉₁. Blank groups were injected endotoxin tested phosphate saline (DPBS). The hemocytes were sampled at 1, 3, 6, 9 and 12 h post injection. The mRNA expression was analyzed by quantitative real time PCR. **(A)** Two doses of DnaK were given to *L. vannamei*: a high dose (5 µg/shrimp) and a low dose (0.05 µg/shrimp). **(B)** Equivalent doses of DnaK₄₄₂₋₄₉₁ were applied to *L. vnnamei* depending on the molecular mass. A high dose of DnaK₄₄₂₋₄₉₁ is 0.3 µg/shrimp and 0.003 µg/shrimp was used as a low dose. Bars indicate standard errors. Significant differences between the treatment and blank at corresponding time points are indicated by ***($P < 0.001$), **($P < 0.01$).

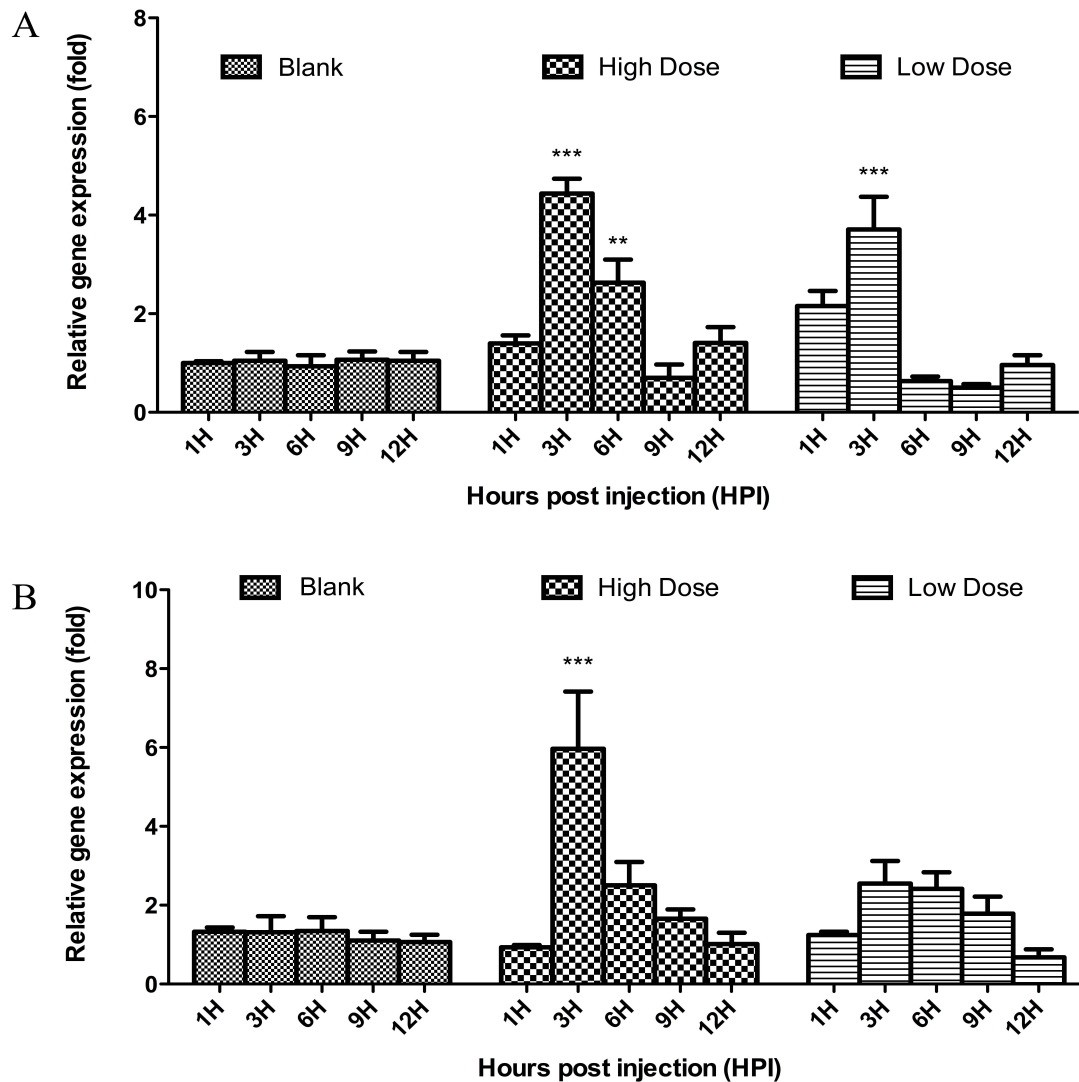


Figure 3.2 Expression of prophenoloxidase-2 (proPO-2) mRNA in *L. vannamei* injected with the DnaK or the 50 amino acid peptide DnaK₄₄₂₋₄₉₁. *L. vannamei* were injected with either recombinant DnaK or DnaK₄₄₂₋₄₉₁. Blank groups were injected with endotoxin tested phosphate saline (DPBS). The hemocytes were sampled at 1, 3, 6, 9 and 12 h post injection. The mRNA expression was analyzed by quantitative real-time PCR. **(A)** Two doses of DnaK were given to *L. vannamei*: a high dose (5 µg/shrimp) and a low dose (0.05 µg/shrimp). **(B)** Equivalent doses of DnaK₄₄₂₋₄₉₁ were applied to *L. vannamei* depending on the molecular mass. A high dose of DnaK₄₄₂₋₄₉₁ is 0.3 µg/shrimp and 0.003 µg/shrimp was used as a low dose. Bars indicate standard errors. Significant differences between the treatment and blank at corresponding time points are indicated by ***($P < 0.001$), **($P < 0.01$).

4. Discussion

Accumulating evidence suggests that HSP70 is an activator of the innate immune system (Pockley, 2005; Tsan, 2004; Wallin et al., 2002), e.g., stimulating cells of the innate immune system. The first observations using recombinant HSP70 showed that HSP70 was able to elicit cytokines production such as IL-1 or TNF- α from human monocytes or mice macrophages *in vitro* (Basu et al., 2002; Galdiero et al., 1997). Moreover, it has been demonstrated that the HSP70 innate-immune activating mechanism is mediated through the Toll-like receptor 2 and Toll-like receptor 4, leading to pro-inflammatory cytokines up-regulation via the NF- κ B pathway in human monocytes (Asea, 2002).

HSP70 has been shown to bind to other proteins and other molecules such as endotoxin LPS, LPS associated molecules, etc., by the protein-binding domain. Other pathogen associated molecular patterns (PAMPs) have been found to be associated with HSP70 as well. Hence, the capacity of HSP70 to activate the immune system has been questioned as endotoxin and other microbial contaminations from eukaryotic vector can be present in the protein preparation and the idea has been put forward that the immune-activating ability of HSP70 is due to endotoxin contamination (Bausinger et al., 2002; Gao, 2002). In our recombinant DnaK preparation, the endotoxin content was measured to be 0.45EU/mg DnaK. The maximum dose of endotoxin administered with the recombinant DnaK is considerably less than the dose that induced the immune response in crustaceans (Okumura, 2007; Söderhäll and Hall, 1984). To further substantiate the idea that the DnaK itself is inducing an immune response, the peptide DnaK₄₄₂₋₄₉₁ was chemically synthesized. In this peptide preparation endotoxin content is undetectable (<0.005 EU/ml). As blank and in the whole procedure, nuclease-, pyrogenic-free apparatus and endotoxin tested balanced salt solution (DPBS, endotoxin content <0.03EU/ml) were used. Two vital immune-related genes, transglutaminase (TGase) and prophenoloxidase (proPO) were used as indicators for the immune response. We showed that both recombinant DnaK and the chemically synthesized DnaK epitopic peptide DnaK₄₄₂₋₄₉₁ induced the immune response in a very similar way. These results strongly suggest that in shrimps, DnaK as such, can stimulate the immune system,

and that contaminants are not solely responsible for the observed proPO and TGase gene up-regulation.

DnaK consists of three functionally distinct domains: an N-terminal 44-kDa ATPase domain, an 18-kDa peptide-binding domain, and a C-terminal 10-kDa lid domain (Todryk et al., 2003; Zhu et al., 1996). It has been shown that the N-terminal failed to induce the pro-inflammatory cytokines, while the peptide-binding domain and C-terminal domains resulted in the enhancement of chemokine production and dendritic cells maturation (Wang et al., 2002). Wheeler et al. (2011) demonstrated that the C-terminus of human HSP70 is a more potent inducer of NF- κ B activity and tumor necrosis factor- α (TNF α) expression compared to the N-terminus. Moreover, using dendritic cells, Wang et al. (2005) identified the cytokine-stimulating epitope (peptide 407-426) of HSP70. In crustaceans, in a more limited study the peptide-binding domain and C-terminal domain successfully primed the proPO gene expression and protected *Artemia* from *Vibrio campbellii* infection (Baruah et al., 2013). In line with literature data on the active region of DnaK, DnaK₄₄₂₋₄₉₁ dramatically induced two immune-related genes, TGase-1 and proPO-2. The up-regulation in *L. vannamei* strongly suggests that this fragment peptide is the most active part of the DnaK protein. On the other hand, it is noticeable that gene expression induced by the recombinant full DnaK protein is stronger than the fragment peptide DnaK₄₄₂₋₄₉₁. Probably because DnaK₄₄₂₋₄₉₁ does not cover the full active region of DnaK, or the recombinant DnaK might still carry a small amount of DnaK-associated compounds that possess synergistic activity.

DnaK is a HSP70 homologue, which is derived from Gram-negative bacteria. A single marine bacterial cell has been reported to contain 10^{-14} – 10^{-13} g protein (Lee and Fuhrman, 1987; Simon and Azam, 1989). HSPs may constitute about 5% of the total protein under non-stressed conditions (Srivastava, 2002). Considering the percentage variety of HSP70 in total HSPs, the high dose of DnaK (5 μ g) represents about 10^7 – 10^9 bacterial cells, while the low dose of DnaK (0.05 μ g) represents about 10^5 – 10^7 CFU respectively. Phuoc et al. (2008, 2009) evaluated the *L. vannamei* mortality infected by *Vibrio* via intramuscular injection. In those studies, no linear relationship between *Vibrio* dosage and mortality was found: with a dosage below 10^7 CFU resulting in little mortality,

while the dosage above 10^7 CFU producing 100% mortality within 24 h post infection. In our research, considering the approximate calculations above, the low dose of DnaK corresponds to a *Vibrio* injection sitting on the borderline between a non-lethal and lethal dosage, while the high dosage corresponds to a lethal injection of *Vibrio* in shrimps. We found that the low dose of both DnaK and DnaK₄₄₂₋₄₉₁ induced a stronger immune response in *L. vannamei* compared to the high dose. This reverse dose-response relationship hints that, due to some unknown mechanism, a potential immune-depressive effect of DnaK cannot be ignored during immune function exploration of DnaK.

Currently, there is an argument that the HSPs are no danger associated molecular patterns (DAMPs), but rather 'DAMPERS' (Eden et al., 2011) as in the case of sterile tissue damage in higher eukaryotes. The extracellular HSP70 would dampen the immune activation and keep homeostasis. In our study, the microbial HSP70 induced the immune response in *L. vannamei* and this seems to prove that the microbial HSP70 *per se* is a danger signal to the immune system of *L. vannamei*. This would imply that HSP70 might have a different role depending on the host considered.

In conclusion, it was established for the first time that the HSP70 homologue DnaK and its immune-activating epitope peptide DnaK₄₂₁₋₄₉₂ are able to significantly stimulate genes of the immune system of *L. vannamei*. These findings raise the hypothesis that bacterial-derived HSP70 confers an alarm signal to the host immune system activating proPO-2 and TGase-1. Further experiments, for instance using standardized challenge tests, will be needed to verify whether HSP70 homologs can be explored in a holistic approach towards the control of disease in crustaceans.

CHAPTER 4

BACTERIAL HSP70 (DNAK) INDUCES IMMUNE
RELATED GENE EXPRESSION IN *LITOPENAEUS VANNAMEI*

Abstract

In aquatic animals, HSP70 has been proven to play an important role in many aspects, especially in the development of inflammation and the specific and non-specific immune responses to bacterial and viral infection. Whiteleg shrimp (*Litopenaeus vannamei*) as main economic aquaculture species is susceptible to infectious diseases, which cause huge loss in shrimp production every year. In our research, we injected specific pathogen free (SPF) shrimps with recombinant HSP70 homologue DnaK. The dynamics of serine proteinase (SP), prophenoloxidase (proPO), transglutaminase (TGase), penaeidins (PENs) and endogenous HSP70 (lvHSP70) gene expression were monitored via quantitative Real-time PCR (qRT-PCR). The serine proteinase (SP), penaeidin-3 (PEN-3) and endogenous HSP70 (lvHSP70) were not significantly induced by DnaK injection. However, the transglutaminase-1 (TGase-1) was dramatically up-regulated by a low dose of DnaK (0.05 µg/shrimp) to 60-fold at 6 hour post injection (hpi). The prophenoloxidase-2 (proPO-2) was induced 5-fold during 3 h by both high dose and low dose of DnaK. The PEN-2 and PEN-4 expression slightly decreased after the first 6 hours then increased. Those results suggested that microbial HSP70 is able to induce the immune response in *L. vannamei* through certain immune related genes.

1. Introduction

Heat Shock Proteins (HSPs) are the most abundant and ubiquitous proteins in single-cell organisms, invertebrates and vertebrates. HSPs normally constitute up to 5% of the total intracellular proteins (Srivastava, 2002). Their expression is up-regulated by various physiological stress factors, such as high temperature, toxins, oxidative conditions, glucose deprivation and microbial infection that potentially lead to host's cell damage (Srivastava, 2002; Stewart and Young, 2004). HSPs are mostly regarded as molecular chaperons that regulate a range of essential housekeeping and cytoprotective functions (Tsan, 2004). Under certain physiological circumstances, intracellular HSPs can be released *via* passive necrosis or active secretion. Evidence is suggesting that in higher animals, extracellular HSPs can elicit cytokine production and adhesion molecule expression in a range of cell types; they also can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions. These findings indicated that HSPs could be immune-regulatory agents with potent and widely-applicable therapeutic uses (Srivastava, 2002; Todryk et al., 2003).

Litopenaeus vannamei is the most widely cultured shrimp species in the world. As high mortality due to infectious diseases emerged, a key constraint to the sustainable production of shrimp farming is to better understand and control the shrimp's immune competence. Like other crustaceans, immune defense in *L. vannamei* solely relies on the innate response that has partly been elucidated via biochemical, proteomic and genomic approaches in recent decades (Rowley and Pope, 2012). The invertebrate immune system comprises both cellular and humoral immunity that are intimately linked. The main immune defense cells in crustaceans are the blood cells (hemocytes) whilst the hepatopancreas is responsible for the biosynthesis of some humoral factors (Cerenius et al., 2010; X. Lin and I. Söderhäll, 2011). Upon the pathogen's invasion through the cuticle or gastrointestinal tract, they will contact directly with hemocytes in the underlying haemocoel. Subsequent immune defense such as prophenoloxidase activating cascade, clotting cascade, antimicrobial peptide (AMP) secretion, phagocytosis and pathogen encapsulation will be triggered (Johansson et al., 2000; Tassanakajon et al., 2013).

As a member of the most studied and highly conserved HSP70 family, it has been proven that the microbial HSP70 homologue DnaK is able to boost the immune system of aquatic animals and protect them from infectious diseases (Lin et al., 2011; Ryckaert et al., 2010; Sung et al., 2009). Nevertheless, the mechanism behind the immune-stimulatory phenomenon hasn't been completely elucidated yet.

This study focuses on the immune response in *L. vannamei* upon injection of contaminant-free heat shock protein 70 homologue DnaK (HU et al., 2014), by looking at hemocytes' gene expression of several immune-related genes including serine proteinase (SP), prophenoloxidas (proPO), transglutaminases (TGase), penaeidins (PENs) and endogenous HSP70 (lvHSP70). The results showed that within 12 h post DnaK injection proPO-2 and TGase-1 were strongly regulated, a phenomenon that was absent for PEN-3, SP and lvHSP70. This suggested that microbial HSP70 (DnaK) is able to stimulate the immune system of *L. vannamei* in a gene-specific way.

2. Materials and methods

2.1 Production of recombinant DnaK*

The *E.coli* strain E_{native} (overexpressing DnaK with a hexahistidine-tag) was constructed and described previously (Baruah et al., 2013). These bacteria were stored in 40% glycerol at -80°C, allowed to thaw, and grown on Luria-Bertani (LB) agar containing kanamycin at 37°C for 24 h. One colony was inoculated in LB broth and grown on a rotary shaker to log phase (OD₆₀₀ 0.4-0.6). Overproduction of DnaK was induced by adding L-arabinose (0.5 mg ml⁻¹) at 37°C for 4 hours. After induction, the bacteria were transferred into sterile tubes, centrifuged at 2200g for 15 min, suspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen). Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in a mini beadsbeater (Biospec, USA) and subsequently centrifuged at 2200 g for 1 min at 4 °C. The supernatant protein was taken for DnaK isolation.

For DnaK isolation, Dynabeads® (Dynabeads® His-Tag Isolation & Pull down, Invitrogen) were used according to the recommended protocol. Briefly, cell

* Repetitive description of previous chapter

lysate was incubated with Dynabeads® for 10 minutes. The Dynabeads® were washed with fresh DPBS 4 times. Finally the DnaK was eluted into elution buffer from the Dynabeads®. The elution buffer was exchanged to DPBS using the Amicon® Ultra Centrifugal Filter (Millipore) according to the manual. The isolated DnaK was stored at -80°C as aliquots for future application.

2.2 Recombinant DnaK analysis*

The isolated recombinant DnaK concentration was determined by the Bradford assay (Bio-Rad). 1 µg DnaK sample was then combined with loading buffer, vortexed, heated at 95°C for 5 min and electrophoresed in 10% SDS-PAGE gels. Gels were either stained with Bio-safe Coomassie stain (Bio-Rad) or transferred to polyvinylidene fluoride membranes (Bio-Rad) for antibody probing. Membranes were incubated with blocking buffer, 50 ml of 1× Phosphate Buffer Saline (PBS) containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin (Sigma), for 60 min at room temperature followed by monoclonal antibody 8E2/2, raised in mouse to DnaK, at the recommended dilution of 1:1000 (Stressgen Bioreagents), as primary antibody. Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO). Detection was done with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) in association with 0.01% (v/v) H₂O₂ in 0.1 M Tris-HCl (pH 7.6). Both SDS-PAGE and Western Blot showed a highly purified single band (appendix I).

The endotoxin lipopolysaccharide (LPS) content of the recombinant DnaK was measured with a chromogenic endotoxin detection kit (GenScript, NewJersey, USA). The endotoxin content of the recombinant DnaK stock was 0.45 EU/mg DnaK.

* Repetitive description of previous chapter

2.3 Experimental animals and conditions

Specific-pathogen-free (SPF) *L. vannamei* were imported from Sy-Aqua SiamCo. Ltd. Bangkok 10110, Thailand. Animals were certified to be free of Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) by the Thai Department of Fisheries. Batches of shrimp arrived at the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), Ghent University, as postlarvae (PL8–12). They were kept in a recirculation system at a water temperature of 28–29°C, 34 g l⁻¹ salinity and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high-performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Hemolymph of *L. vannamei* was examined randomly by plating on Marine agar and no growth was observed. Shrimps for experimental purpose (MBW = 15.6 ± 1.3 g) were housed individually in covered 10 L aquaria, filled with Instant Ocean® seawater prepared with distilled water at a salinity of 35 g l⁻¹, provided with constant aeration and maintained at 27 ± 1°C by air heaters. Shrimps were acclimated under the latter conditions for 3 days before subsequent treatment. Feeding was skipped for 24 h prior to the injection. Dim light had been applied during the whole culture and experimental process.

2.4 Recombinant DnaK treatment

Two doses of recombinant DnaK protein were injected in the shrimp, a high dose (5 µg) and low dose (0.05 µg) per animal (The DnaK dosage calculation is elucidated in appendix II). DnaK was dissolved in 100 µl Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium and no magnesium, Gibco®, Invitrogen) and then intramuscularly injected in the tail. As control, DPBS was injected. The hemolymph was collected at 5 time points post injection, 1, 3, 6, 9 and 12 hours post injection (hpi). Three animals from each treatment were sacrificed at every time point.

2.5 Total RNA isolation and cDNA synthesis*

Hemolymph (200 µl) was collected from each shrimp using a 1 ml syringe with 20G needle containing 300 µl of precooled anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4). After centrifugation of 250 g at 4°C for 5 minutes, the hemocytes pellets were quick frozen in liquid nitrogen and then preserved at -80°C for RNA extraction. RNA sample from different shrimps were processed and analyzed individually.

Total RNA was extracted from hemocytes using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Fermentas) to remove genomic DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 0.5 µg total RNA using the RevertAid™ 6H minus First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions.

2.6 Quantitative real-time PCR (qRT-PCR) analysis

The immune related genes, prophenoloxidase, serine proteinase, transglutaminase, penaeidins and endogenous heat shock protein 70 (lvHSP70) mRNA expression in *L. vannamei* were analyzed by qRT-PCR using a pair of specific primers designed on the basis of several *L. vannamei* cDNA sequence (**Table 4.1**, GeneBank accession no.). The β-actin was used as reference gene (the reference gene selection see appendix III). Real-time PCR was performed in an ABI StepOne™ Real Time System thermal cycler (Applied Biosystem) in a total volume of 20 µl containing 10 µl of 2x SYBR Green qRT-PCR Master mix (Applied Biosystems), 0.5 µl (each) forward/reverse primers (10 µM), 5 µl of cDNA template and 4 µl nuclease-free water (Sigma). The thermal cycle parameters used for the real-time amplification were: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 30 s and elongation at 60°C for 30 s. Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of

* Repetitive description of previous chapter

untargeted fragments. Each cDNA sample was performed in two replicates. Data acquisition was recorded with the StepOne™ software (v 2.2.2, Applied Biosystem) at the end of each elongation step.

Table 4.1: Gene bank accession No. and primer sequences used in qRT-PCR

Gene	Gene Bank Accession No.	Primers
β-actin	AF300705.2	F: GATGTGTGACGACGAAGTAG R: GATACCTCGCTTGCTCTGG
Prophenoloxidase 1 (proPO-1)	AY723296.1	F: AACTCCATTCCGTCCGTCTG R: GGCTTCGCTCTGGTTAGGAT
Prophenoloxidase 2 (proPO-2)	EU373096.1	F: CTCAGCGTGAACTCGCCTTA R: CCTGCTCAGTGTACGGTCT
Transglutaminase 1 (TGase-1)	EF081004.1	F: GAGCTTCAAGATCGAGGATCGA R: GCTGGTGTTTCGTAGCGGTTATC
Transglutaminase 2 (TGase-2)	EU164849.1	F: TCGGAGAAACCAAGAGCATGA R: ATAAGATCGTTCGGACCCACA
Penaeidin 2 (PEN-2)	Y14925	F: TCGTGGTCTGCCTGGTCTT R: CAGGTCTGAACGGTGGTCTTC
Penaeidin 3 (PEN-3)	Y14926	F: CACCCTTCGTGAGACCTTTG R: AATATCCCTTTCCCACGTGAC
Penaeidin 4 (PEN-4)	AF390147	F: GCCCGTTACCCAAACCATC R: CCGTATCTGAAGCAGCAAAGTC
Serine proteinase (SP)	AY368151	F: ACGTTCTCAGACTGGTCACAC R: TATGTAAGGCGCGTCGTTCTC
Endogenous Heat Shock Protein 70 (IvHSP70)	AY645906.1	F: CTACTCCTGCGTGGGTGTGTT R: TCAGTCGCTTGGCATCGAATAC

2.7 Real-time PCR data analysis

The real-time PCR was validated by amplifying dilution series of cDNA synthesized from 0.5 µg of RNA isolated from hemocytes samples. These cDNA

dilutions were amplified by real-time PCR using gene specific primers. ΔC_T (average C_T value of target – average C_T value of β -actin) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 1 for all of the target genes. Therefore, the amplification efficiency of reference and the target genes is equal. The expression of the target genes was normalized to the endogenous control β -actin by calculating ΔC_T (Livak and Schmittgen, 2001):

$$\Delta C_T = C_{T, \text{target}} - C_{T, \beta\text{-actin}}$$

and expressed relative to blank group (calibrator) by calculating $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T, \text{calibrator}}$$

The relative expression was then calculated as

$$\text{Relative expression (fold)} = 2^{\Delta\Delta C_T}$$

2.8 Statistics

Data were analyzed by two-way analysis of variance (ANOVA) with post-hoc Bonferroni's correction (GraphPad Prism 5.0; GraphPad Software) to determine significance. All data were expressed as mean \pm SEM. Values were considered significantly different if $p < 0.05$.

3. Results

3.1 Prophenoloxidase (proPO) activating cascade gene mRNA expression

The prophenoloxidase-activating cascade (proPO-AS) is the most studied immune defense mechanism in crustaceans. The proPO-AS is triggered by multiple factors, such as pathogen associated molecular patterns (PAMPS), tissue damage and possibly by enzymes released from pathogens (Söderhäll et al. 1998). In our study, serine proteinase, an upstream proteinase responsible for processing prophenoloxidase into phenoloxidase, proPO-1 and proPO-2 expression were measured via qRT-PCR. Both a high dose (5 μ g) and a low dose (0.05 μ g) of DnaK were, upon injection, not able to regulate serine proteinase expression at subsequent sampling points (1, 3, 6, 9, 12 h post injection, hpi) during a 12 hours interval (**Fig.4.1A**). The mRNA expression of prophenoloxidase-1 (proPO-1) was down regulated at 1-6 hpi's by a high dose of DnaK ($p < 0.05$, **Fig.4.1B**). However, both the high dose and low dose of DnaK

induced the expression of prophenoloxidase-2 (proPO-2) (**Fig.4.1C**, data were presented in chapter 3) producing an expression peak at 3 hpi (4-fold, $p<0.001$), which subsided afterwards.

3.2 Transglutaminase (TGase) mRNA expression

Up to date, two TGase genes, producing enzymes involved in the shrimp clotting system, have been cloned and sequenced in *L. vannamei* and named TGase-1 and TGase-2 respectively. Surprisingly, the measurement showed that TGase-1 expression in *L. vannamei* hemocyte (**Fig.4.2A**, data was presented in chapter 3), upon injection with the low dose of DnaK, sharply increased to 60-fold compared to the control ($p<0.001$) at 6 hpi. Meanwhile, the injection of a high dose of DnaK resulted in about 10-fold increase of TGase-1 ($p<0.001$). On the other hand, the TGase-2 expression (**Fig.4.2B**) in shrimp treated with a high dose of DnaK did not exhibit a significant change, while shrimps injected with a low dose of DnaK showed two significant increases at 1 hpi and 12 hpi respectively ($p<0.05$).

3.3 Penaeidins (PENs) mRNA expression

Expression of penaeidins (PEN-2, PEN-3, PEN-4) was measured (**Fig. 4.3**). A low dose of DnaK up-regulated the PEN-2 (**Fig. 4.3A**) to 1.6-fold at 1 hpi ($p<0.05$). The PEN-2 expression decreased at 6 hpi (high dose) and 9 hpi (low dose). PEN-3 (**Fig. 4.3B**) expression fluctuated and didn't show any significant regulation. Both high dose and low dose of DnaK injection resulted in a decrease of PEN-4 (**Fig.4.3C**) at 6hpi, and were subsequently up-regulated. At 12 hpi, PEN-4 was up-regulated 1.5-fold by injection of a low dose of DnaK ($p<0.001$).

3.4 Endogenous HSP70 (lvHSP70) expression

To investigate the putative effect of exogenous DnaK on endogenous HSP70 expression in *L. vannamei*, the endogenous HSP70 expression was also evaluated. The results (**Fig.4.4**) showed that the high dose of DnaK slightly up-regulated endogenous HSP70 expression at 6 hpi (1.5-fold, $p<0.05$). A low dose of DnaK stimulated the endogenous HSP70 to 1.8-fold at 1 hpi ($p<0.001$) relative to the

control, and the endogenous HSP70 expression fluctuated and no significant regulation was observed.

4. Discussion

Earlier studies have shown that HSPs, especially members of the HSP70 family, play a vital role in innate immunity (Gaston, 2002; Wallin et al., 2002). Intensive research has been done using HSPs as immune therapy to cure cancer, autoimmune and infectious diseases (Todryk et al., 2003; Vinokurov et al., 2011). In order to figure out the immune effect of HSP70, the mostly applied method is to use recombinant HSP70 in *in vivo* and *in vitro* immune response tests. However, as most of the recombinant HSPs are produced by *Escherichia coli* expressing HSP cDNAs, the final preparations might be contaminated with bacterial components. In vertebrate innate immunity, the HSPs have similar cytokine inducing effect with those of pathogen-associated molecules (PAMPs), such as lipopolysaccharide (LPS), bacterial lipoprotein and so on. On the other hand, even though no acquired immunity has been found in invertebrates, their immune response can be triggered by bacterial components *via* multiple pathways such as prophenoloxidase cascade and clotting cascade (Söderhäll and Cerenius, 1998; Theopold et al., 2004). In our research, we applied endotoxin-tested recombinant DnaK and a synthetic DnaK fragment peptide DnaK₄₄₂₋₄₉₁ and found that the immune response was highly consistent (Hu et al., 2014). In this way, we are able to eliminate the interference of microbial contaminants with the *L. vannamei* immune system.

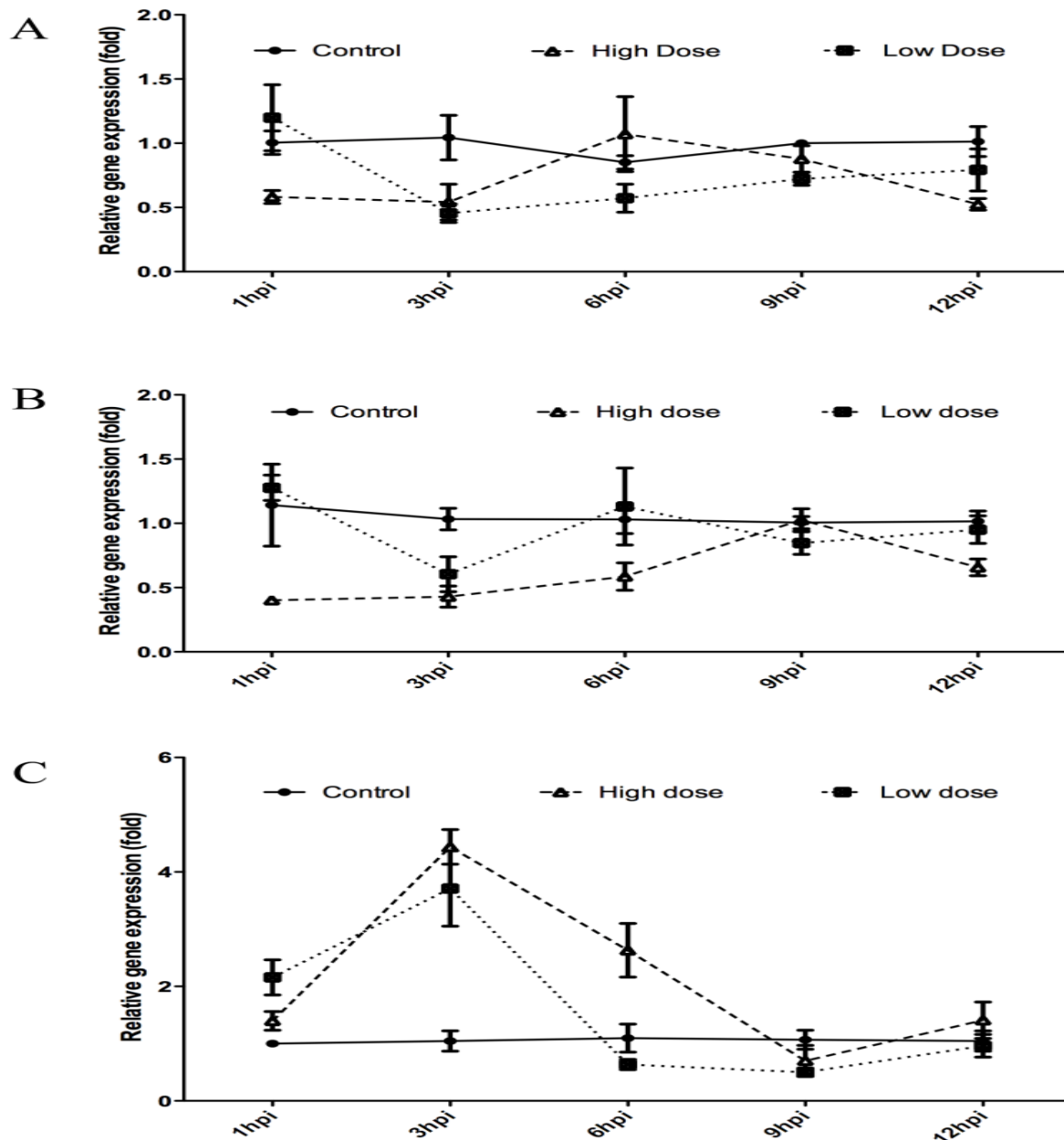


Figure 4.1 *In vivo* expression of (A) serine proteinase, (B) prophenoloxidase-1, (C) prophenoloxidase-2 in *L. vannamei* injected with recombinant DnaK. Control groups were injected with endotoxin tested phosphate saline (DPBS). Hemocytes of 3 shrimps were sampled at each time point (1, 3, 6, 9 and 12 hpi). Total mRNA was extracted from the hemocytes and cDNA was subsequently synthesized. The mRNA expression was performed in 2 replicates by quantitative real-time PCR. Two doses of DnaK were given to *L. vannamei*: a high dose (5 μ g/shrimp) and a low dose (0.05 μ g/shrimp). Bars indicated standard error.

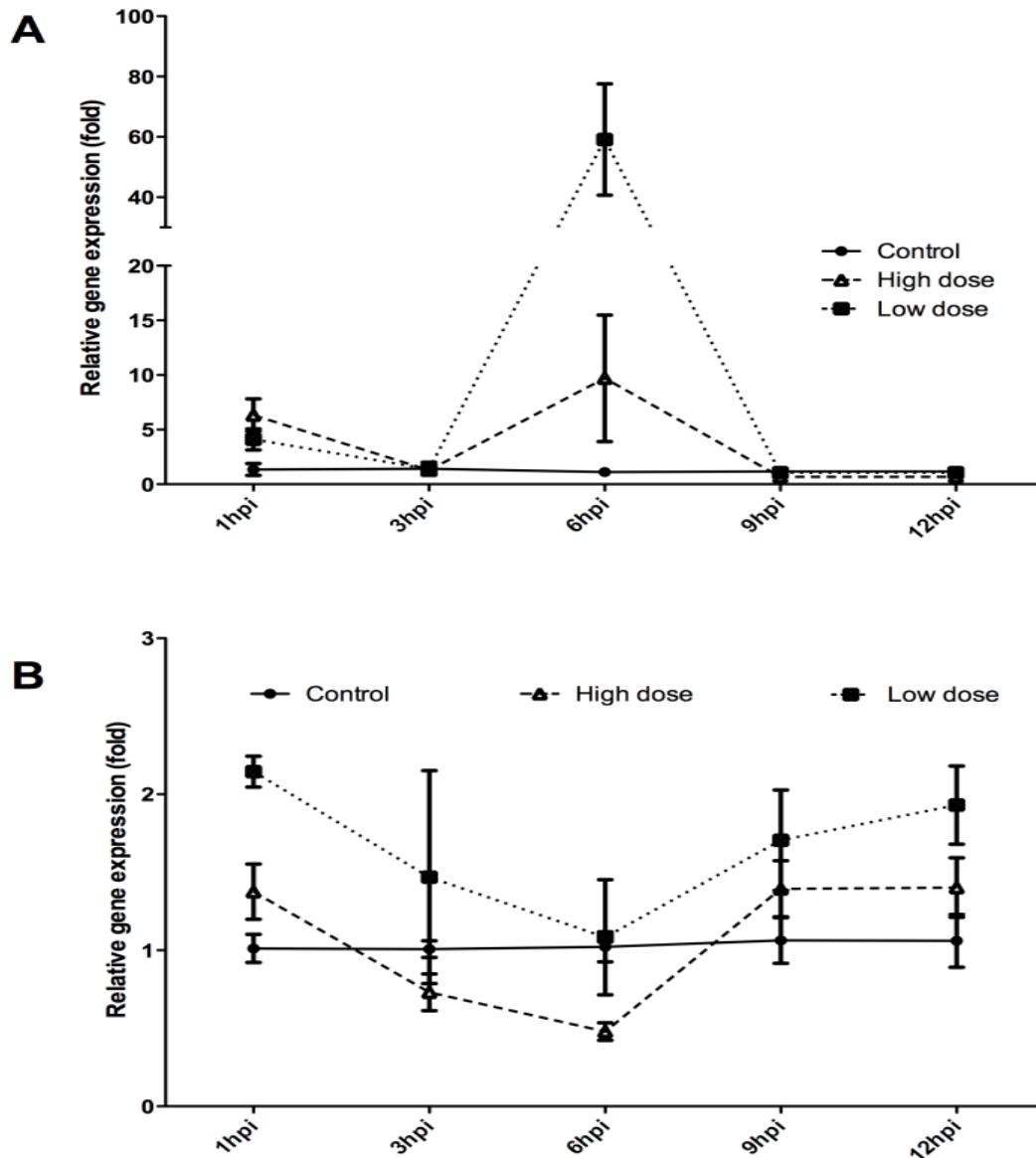


Figure 4.2 *In vivo* expression of (A) transglutaminase-1, (B) transglutaminase-2 of *L. vannamei* injected with recombinant DnaK. Control group were injected endotoxin tested phosphate saline (DPBS). Hemocytes of 3 shrimps were sampled at each time point (1, 3, 6, 9 and 12 hpi). Total mRNA was extracted from the hemocytes and cDNA was subsequently synthesized. The mRNA expression was performed in 2 replicates by quantitative real-time PCR. Two doses of DnaK were given to *L. vannamei*: a high dose (5 μ g/shrimp) and a low dose (0.05 μ g/shrimp). Bars indicate standard errors.

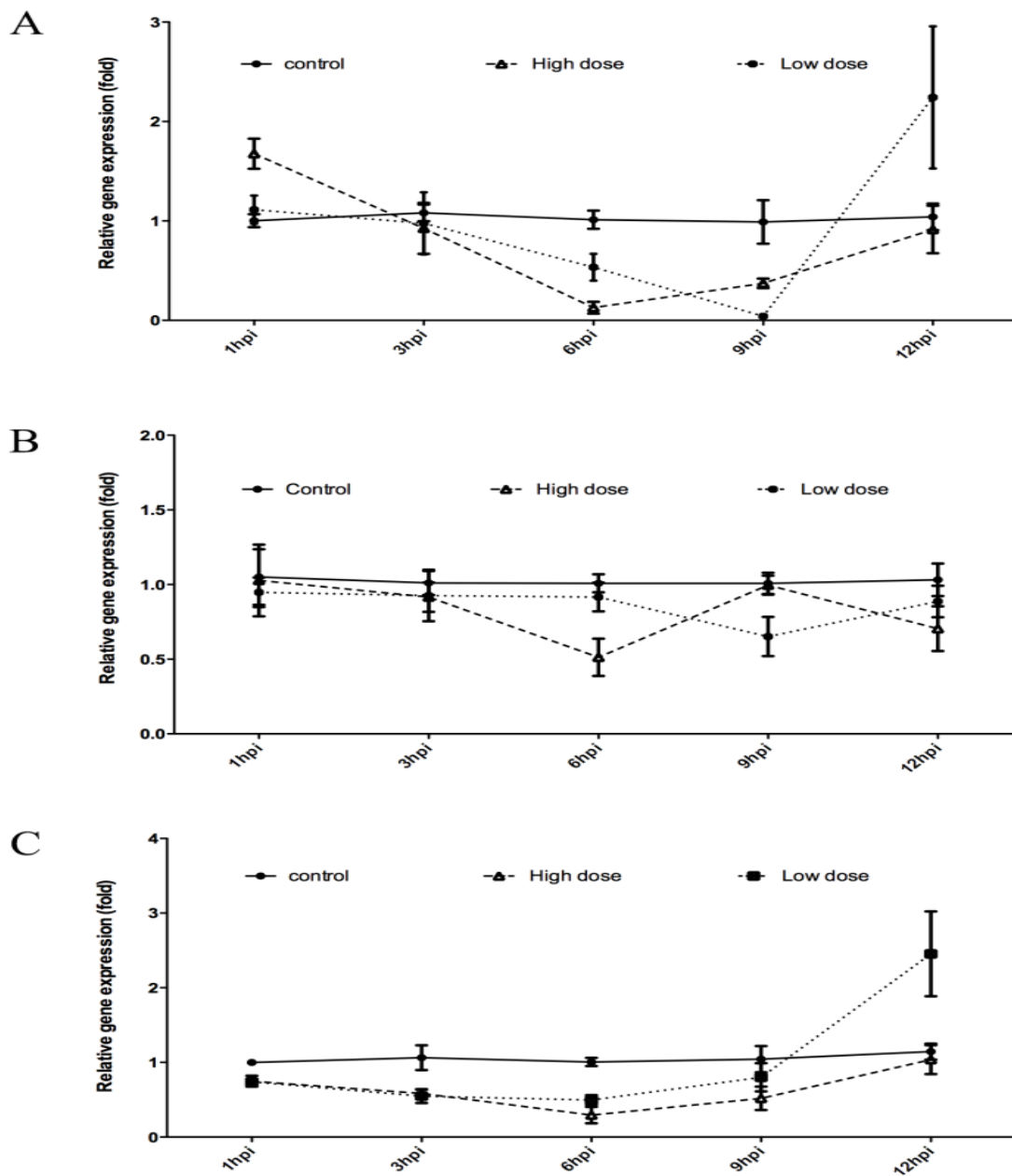


Figure 4.3. *In vivo* expression of (A) penaeidin 2, (B) penaeidin 3, (C) penaeidin 4 in *L. vannamei* injected with recombinant DnaK. Hemocytes of 3 shrimps were sampled at each time point (1, 3, 6, 9 and 12 hpi). Total mRNA was extracted from the hemocytes and cDNA was subsequently synthesized. The mRNA expression was performed in 2 replicates by quantitative real-time PCR. Two doses of DnaK were given to *L.vannamei*: a high dose (5 μ g/shrimp) and a low dose (0.05 μ g/shrimp). Bars indicate standard errors.

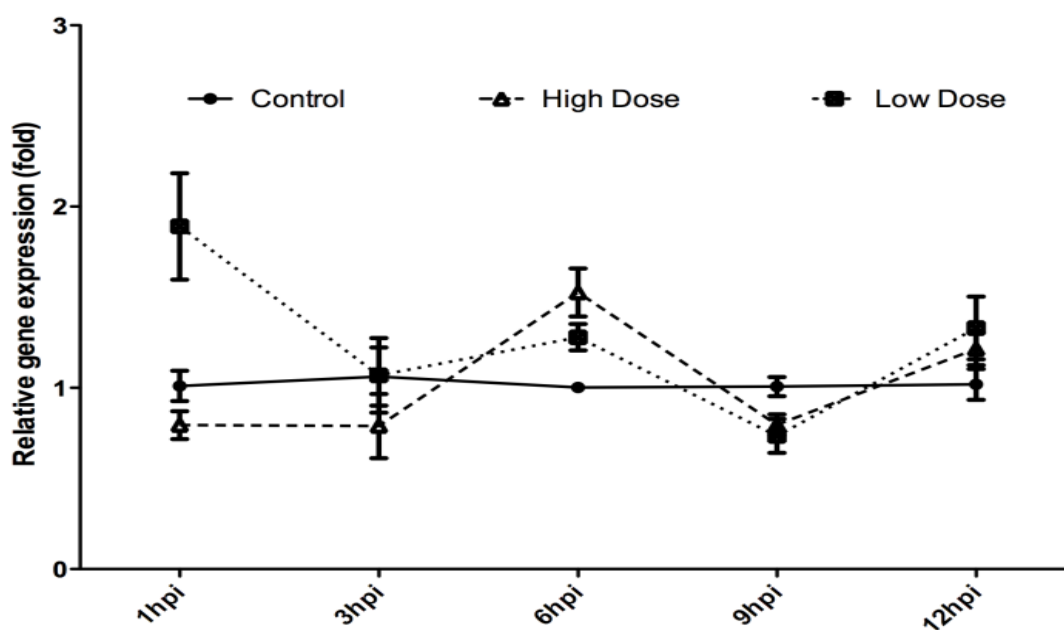


Figure 4.4 *In vivo* expression of endogenous Heat Shock Protein 70 (HSP70) in *L. vannamei* injected with recombinant DnaK. Hemocytes of 3 shrimps were sampled at each time point (1, 3, 6, 9 and 12 hpi). Total mRNA was extracted from the hemocytes and cDNA was subsequently synthesized. The mRNA expression was performed in 2 replicates by quantitative real-time PCR. Two doses of DnaK were given to *L.vannamei*: a high dose (5 μ g/shrimp) and a low dose (0.05 μ g/shrimp). Bars indicate standard errors.

In crustaceans immunity, TGase has been documented to be involved in blood coagulation, which is a conserved defense mechanism among invertebrates (Hall et al., 1999; Maningas et al., 2013; R. Wang et al., 2001). It is released from the hemocytes in response to tissue damage or intrusion of microbial pathogens and then catalyzes the polymerization of the plasma clotting protein to achieve clotting (Hall et al., 1999; R. Wang et al., 2001; Z. Wang et al., 2010). Z. Wang et al. (2010) found out that in *Drosophila* the TGase activity accumulated on the pathogen surface, entrapping them into the clot. Additional circumstantial evidence for an immunological role of the TGase comes from kuruma shrimps (*Marsupenaeus japonicus*). TGase-silenced shrimps suffered higher mortality when they were challenged with white spot virus and *Vibrio penaeicida* (Lin et al.,

2008; Maningas et al., 2008). These results suggest that TGase plays an important role in hemolymph clotting and anti-microbial infection immune response in invertebrate immunity. Moreover, in *Pacifastacus leniusculus*, knocking out the TGase gene led to morphological changes of hemocytes (Horvath et al., 2008; Lin et al., 2008). This finding indicates that the TGase affects hematopoiesis in addition to coagulation. Our results showed that the low dose of recombinant DnaK induced a strong TGase-1 up-regulation at 6 h post injection. Purposely designed experiments will be needed to unravel how the apparent function of TGase in hematopoiesis and coagulation can be reconciled with its activation by DnaK. The knowledge that heat shock proteins are often cell-membrane associated (Cerenius and Söderhäll, 2004; Horvath et al., 2008; Söderhäll and Cerenius, 1998) , potentially making them a target for TGase during the coagulation process, might be instrumental.

The Prophenoloxidase (proPO) Activating System (proPO-AS) is also an important part of the crustaceans immune system and has been proven to be an important immune defense mechanism against several pathogens including bacteria and parasites (Cerenius and Söderhäll, 2004; Ross et al., 2003; Söderhäll and Cerenius, 1998). Until now two proPO genes have been discovered in *L. vannamei*. Those two proPO genes exhibited different expression patterns upon DnaK injection in our study. The proPO-2 was significantly up-regulated during the first three hours post injection and down-regulated afterwards, while the proPO-1 expression fluctuated and no significant differences were observed. This suggested that the different proPO genes might have a different function in the immune response to recombinant DnaK. In most invertebrates studied, there is more than one proPO gene. For instance, in fruit flies, there are 3 proPO genes (Ross et al., 2003), 9 proPO genes in the mosquito *Anopheles gambiae* (Christophides et al., 2002) and 2 proPO genes in the crustaceans *Penaeus monodon* (Amparyup et al., 2009). The different proPO genes in mosquito are expressed in different life stages or upon exposure to different pathogens (Muller et al., 1999). In *Penaeus monodon*, it has been noted that the two proPO genes responded differently when the host was exposed by intramuscular injection to White Spot Syndrome virus (WSSV) (Radha et al., 2013). Also in *L. vannamei*, the proPO-1 and proPO-2 behaved differently against microbial

infections (Okumura et al., 2007; Wang HQ et al., 2010; Hsieh et al., 2013; Wang et al., 2008; Yeh et al., 2009). Taken together, these literature data and our findings suggest that member genes of the PO system can be regulated differentially in response to a certain trigger. In crustaceans, it has been discovered that the presence of minute quantities of microbial bacterial components, such as lipopolysaccharide (LPS), β -1,3-glucans and peptidoglycan, can be recognized by pattern recognition proteins. These binding compounds trigger activation of upstream components of the serine proteinase cascade. The terminal proteinase will cleave prophenoloxidase to the catalytically active enzyme phenoloxidase (Amparyup et al., 2013). In our study, the upstream enzyme serine proteinase expression was examined, but no significant regulation was observed at gene transcription level. This is consistent with the previous finding that serine proteinase in *L. vannamei* hemocytes was not stimulated by *Vibrio* inoculation (Okumura, 2007). Moreover, in insects, gene expression of serine proteinase involved in the proPO activating system is not changed by injections of bacteria (Gorman and Paskewitz, 2001; Kim et al, 2002). These results suggest that the stimulation of the serine proteinase activity, if at all, by LPS or bacteria, could be at the post-transcriptional level.

Penaeidins are antimicrobial peptides isolated from crustaceans, which present both anti-bacterial and anti-fungal activities. To date, several penaeidin sequences have been identified from different penaeid shrimp species. In penaeid shrimps, these sequences can be classified into four classes (PEN-2, PEN-3, PEN-4, PEN-5). The whiteleg shrimp *L. vannamei*, PEN-2, PEN-3 and PEN-4 but no PEN-5 were identified by gene cloning. Penaeidins are primarily synthesized in hemocytes and released into the blood circulation in response to microbial stimulation (Destoumieux et al., 2000; Muñoz et al., 2002). In our study, the expression of PEN-2 and PEN-4 reduced at the early stage of DnaK injection (0-6 hours) and then rebound back to normal level. The low dose of DnaK induced PEN-2 and PEN-4 expression at 12 hpi. The results are consistent with previous findings that penaeidin transcripts were reduced in the early hours following stimulation and return to a normal levels after microbial stimulation (Muñoz et al., 2002; Supungul et al., 2004). Munoz (2002) elucidated that in microbial challenged shrimps, the penaeidin-expressing hemocytes migrate to

the site of infection. The down-regulation of penaeidin transcription may result from changes in the circulating hemocyte population.

In 12 hours post injection, the endogenous HSP70 (lvHSP70) fluctuated and not significant differences were observed. There is no significant regulation of lvHSP70 because either the lvHSP70 responded beyond 12 hours, or no direct interaction between exogenous and endogenous HSP70 is taken place.

In this work, we provided evidences that the immunostimulatory effect in *L. vannamei* can be altered by injecting recombinant microbial HSP70 (DnaK). After DnaK injection, proPO-2, TGase-1 expression were dramatically induced, while a minor regulation on PEN-2 and PEN-4 expression was found. It suggests that the bacterial HSP70 could be explored to modulate the expression of immune-related genes of *L. vannamei* potentially involved in mounting a response against infectious diseases.

CHAPTER 5

BACTERIAL HSP70 (DNAK) PRIMES THE IMMUNE
RESPONSE IN *LITOPENAEUS VANNAMEI* AGAINST
VIBRIO CAMPBELLII

Abstract

Similar to other invertebrate, the white-leg shrimp *Litopenaeus vannamei* relies solely on an innate immunity, known to lack adaptive characteristics, to fight against invading pathogens. Nevertheless, in more and more invertebrate species it has been verified that a “trained immunity” exists, which has similarities with the vertebrate adaptive immunity. It is possible that immunostimulants or pathogen-derived products act as “vaccine” against infectious pathogens. The 70 kDa heat shock protein DnaK (an HSP70 homologue), a well conserved and immunodominant molecule derived from prokaryote (*Escherichia coli*), protects brine shrimp *Artemia* against *Vibrio campbellii* by immune priming. Here, we demonstrate that a pre-treatment of *L. vannamei* with the recombinant *E. coli* Hsp70 equivalent DnaK, followed by a non-lethal *V. campbellii* challenge affected the transcription of 3 immune genes, Transglutaminase-1 (TGase-1), prophenoloxidase-2 (proPO-2) and endogenous HSP70 (lvHSP70). These results indicate that bacterial Hsp70 DnaK can modulate immunity in *L. vannamei* against *V. campbellii* infection by priming immune-related gene transcription.

1. Introduction

The Gram-negative bacteria *Vibrio* spp. cause luminous vibriosis and mass mortality in many aquaculture species, such as penaeid shrimp (Austin and Zhang, 2006). Antibiotic-based disease control treatment has been linked to the development of antibiotic-resistant strains, which pose a threat to human health (Smith et al., 2003). To lower the risk of disease outbreak, enhancement of shrimp resistance to pathogens is a promising alternative. Unlike vertebrates, invertebrates, including *L. vannamei*, are reported to possess only innate immunity (Cerenius et al., 2010; Vazquez et al., 2009). Although the innate immunity lacks memory traits, increasing evidence suggests that it could be trained to enhance resistance to a secondary infection (Netea et al., 2011). This enhanced resistance is either caused by a non-lethal dose of the same pathogen (Sadd and Schmid-Hempel, 2006) or a cross-protection triggered by other immune-elicitors such as lipopolysaccharides (LPS) (Moret and Siva-Jothy, 2003).

Heat shock proteins (HSPs) are ubiquitous and highly conserved proteins from prokaryotes to eukaryotes (Pockley, 2003). In recent years, compelling studies suggested that they elicit innate and adaptive immune response against many diseases including microbial infections (Robert, 2003; Srivastava and Amato, 2001). HSPs are categorized into several families based on their approximate molecular weight, such as HSP90, HSP70, HSP60 and small HSPs (Moseley, 2000). Prokaryotic HSP70, DnaK, was suggested to serve as a danger signal to both innate and adaptive immunity (Tobian et al., 2004) and has been used in immune therapies against bacterial infection in mammals (Paliwal et al., 2011). In a model crustaceans species *Artemia*, feeding DnaK overexpressing *E. coli* protects against *Vibrio campbellii* infection (Sung et al., 2009; 2008). Moreover, it has been found that this feeding treatment is also able to prime the prophenoloxidase cascade and protect *Artemia* from subsequent *V. campbellii* challenge (Baruah et al., 2010). On the other hand, overdosing prokaryotic HSP70 appears to be detrimental to hosts (Baruah et al., 2013). Therefore, the immune regulatory mechanism of prokaryotic HSP70 in invertebrate immunity needs to be carefully investigated.

In this study, we primed SPF *L. vannamei* with recombinant bacterial HSP70, DnaK, via intramuscular injection, and then challenged shrimps with a non-

lethal dose of *V. campbellii*. Three vital immune-related genes, which have been shown previously to respond to DnaK administration in *L. vannamei*, transglutaminase-1 (TGase-1), prophenoloxidase-2 (proPO-2) and endogenous HSP70 (lvHSP70) were selected as immune markers (see Chapter 4). Their transcriptions were monitored by quantitative real time PCR (qRT-PCR) at 4 time points within 12 h post DnaK priming (hpp).

2. Materials and methods

2.1 Recombinant DnaK production*

The *E.coli* strain E_{native} (expressing DnaK with a hexahistidine-tag) was described previously (Baruah et al., 2013). The bacteria were stored in 40% glycerol at -80°C, then grown at 37°C for 24 h on Luria-Bertani (LB) agar containing kanamycin then on a rotary shaker to log phase (OD_{600} 0.4-0.6) in LB broth. Overproduction of DnaK was induced by adding L-arabinose (0.5 mg ml^{-1}) at 37°C for 4 hours. After induction, the bacteria were transferred into sterile tubes, centrifuged at 2200g for 15 min, suspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen). Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in a mini beadsbeater (Biospec, USA) and subsequently centrifuged at 2200 g for 1 min at 4 °C. The supernatant protein was taken for DnaK isolation.

For DnaK isolation, Dynabeads® (Dynabeads® His-Tag Isolation & Pull down, Invitrogen) were used according to the recommended protocol. Briefly, cell lysate was incubated with Dynabeads® for 10 minutes. The Dynabeads® were washed with fresh DPBS 4 times. Finally the DnaK was eluted into elution buffer from the Dynabeads®. The elution buffer was exchanged to DPBS using the Amicon® Ultra Centrifugal Filter (Millipore) according to the manual. The isolated DnaK was stored at -80°C as aliquots for future application. The protein analysis is presented in appendix I.

* Repetitive description of previous chapters

2.2 *Vibrio campbellii* culture

The bacterial strain *Vibrio campbellii* (LMG21363) previously stored in 40% glycerol at -80°C, were aseptically streaked on Marine agar plates (Difco Laboratories, MI, USA) plate and then incubated for 24 h at 28°C. Five colonies were subsequently picked up and grown in Marine Broth 2216 (Difco Laboratories, MI, USA) by overnight incubation in a shaker at 28°C. The culture was then transferred to centrifugation tubes and centrifuged at 2200 g for 8 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density (OD) of 600 nm assuming that the bacterial density (Y) equals $8.1362 \times (\text{OD}_{600}) - 0.8516$ (10^8CFU/ml) based on a pre-determined OD-CFU standard curve.

2.3 Experimental animals

Specific-pathogen-free (SPF) *L. vannamei* were imported from Shrimp Improvement Systems (SIS), United States. Animals were tested by PCR for WSSV, TSV, IHNV, YHV, IMNV, and NHP using World Organisation for Animal Health (OIE) approved methodologies and primers. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL8–12). They were kept in a recirculation system at a water temperature of 28–29°C, 34 g l⁻¹ salinity and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high-performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Shrimps were housed individually in covered 10 L aquaria, filled with Instant Ocean® seawater prepared with distilled water at a salinity of 35 g l⁻¹, provided with constant aeration and maintained at 27±1°C by air heaters. Shrimps were acclimated for 3 days before any subsequent treatment. Feeding was skipped for 24 h prior to the injection.

2.4 Experimental design

As shown in **Fig. 5.1** for priming, 0.05 µg recombinant DnaK in 100 µl DPBS (no calcium, no magnesium, Gibco®, Invitrogen) was injected intramuscularly

into *L. vannamei*. The DPBS was used as blank (D-). 1 h post DnaK priming, a non-lethal dose of *Vibrio campbellii* (10^5 CFU/shrimp) was injected (V+). This dose was chosen based on an *in vivo* titration conducted in *L. vannamei* at the same experimental condition (Phuoc et al., 2009a; Phuoc et al., 2009b). The hemolymph was collected at 4 time points post DnaK injection, 1.5, 6, 9 and 12 hours post DnaK priming (hpp). At every time point, three animals from each treatment were sacrificed.

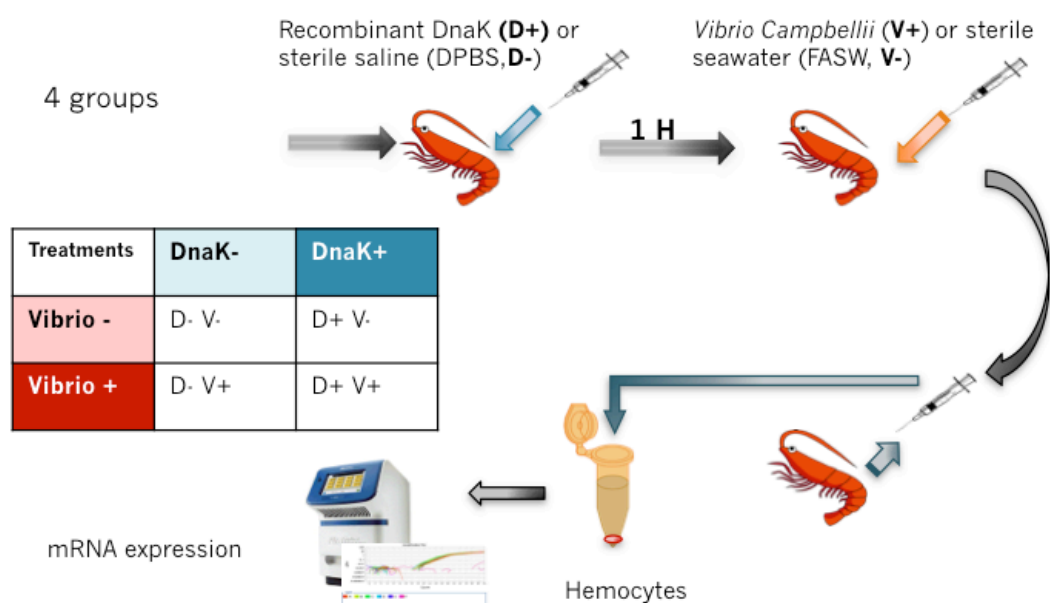


Figure 5.1 Experimental design: Shrimps (*L. vannamei*) were assigned randomly and injected with either recombinant DnaK (0.05 μ g, D+) or sterile saline (DPBS, D-); 1 h post DnaK injection, a non-lethal dose of *Vibrio campbellii* (10^5 CFU, V+) or sterile seawater (FASW, V-) was injected. Hemocytes were sampled 1.5, 6, 9, 12 hours post DnaK injection (hpp). At each time point, 3 shrimps from each treatment were sacrificed. Quantitative real-time PCR (qRT-PCR) was performed to analyze expression of three immune-related genes.

2.5 Immune related gene expression

Hemolymph (200 μ l) was collected from shrimp using a 1 ml syringe with 20G needle containing 300 μ l of precooled anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM tri-sodium citrate, 26 mM citric acid, 10 mM

EDTA, pH 5.4). After centrifugation of 250 g at 4°C for 5 minutes, the hemocyte pellets were quick frozen in liquid nitrogen and then preserved at -80°C for RNA extraction.

Total RNA was extracted from hemocytes using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Fermentas) to remove genomic DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 0.5 µg total RNA using the RevertAid™ 6H minus First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions.

The immune related genes, transglutaminase (TGase-1), prophenoloxidase (proPO-2) and endogenous heat shock protein 70 (lvHSP70) mRNA expression in *L. vannamei* was analyzed by qRT-PCR using a pair of specific primers designed on the basis of several *L. vannamei* cDNA sequence (**Table 5.1**, GeneBank accession no.). Quantitative real-time PCR (qRT-PCR) was performed in an ABI StepOne Real Time System thermal cycler (Applied Biosystem) in a total volume of 20 µl containing 10 µl of 2x SYBR Green qRT-PCR Master mix (Applied Biosystems), 0.5 µl (each) forward/reverse primers (10 µM), 5 µl of cDNA template and 4 µl nuclease-free water (Sigma). The thermal cycle parameters used for the real-time amplification were: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 30 s and elongation at 60°C for 30 s. Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne software (v2.2.2, Applied Biosystem) at the end of each elongation step.

Table 5.1 Gene bank accession No. and primer sequences used in qRT-PCR

Gene	Gene Bank Accession No.	Primers
Transglutaminase-1 (TGase-1)	EF081004.1	F: GAGCTTCAAGATCGAGGATCGA R: GCTGGTGTTTCGTAGCGGTTATC
Prophenoloxidase-2 (proPO-2)	EU373096.1	F: CTCAGCGTGAACCTCGCCTTA R: CCTGCTCAGTGTACGGTCT
<i>L. vannamei</i> HSP70 (lvHSP70)	AY645906.1	F: CTACTCCTGCGTGGGTGTGTT R: TCAGTCGCTTGGCATCGAATAC
β -actin	AF300705.2	F: GATGTGTGACGACGAAGTAG R: GATACCTCGCTTGCTCTGG

The real-time PCR was validated by amplifying of serial dilutions of cDNA synthesized from 0.5 μ g of RNA isolated from hemocytes samples. Serial dilutions of cDNA were amplified by real-time PCR using gene specific primers. Δ CT (average CT value of target – average CT value of β -actin) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 1 for all of the target genes. Therefore, the amplification efficiency of reference and the target genes is equal. The expression of the target genes was normalized to the endogenous control β -actin (RNA polymerase A subunit) by calculating Δ C_T (Livak and Schmittgen, 2001):

$$\Delta C_T = C_{T, \text{target}} - C_{T, \beta\text{-actin}}$$

and expressed relative to control group (calibrator) by calculating $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T, \text{calibrator}}$$

The relative expression was then calculated as

$$\text{Relative expression} = 2^{\Delta\Delta C_T}$$

2.6 Statistics

At each time point, data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test using statistic software GraphPad Prism Programs (GraphPad Prism, San Diego, CA) to determine significance. The

effect from two factors (DnaK, *V. campbellii*) and their interaction were evaluated. Values were considered significantly different if $p < 0.05$.

3. Results

3.1 Transglutaminase (TGase-1) mRNA transcription

Acute TGase-1 response to the immune treatment was monitored within 12 hours after DnaK and blank injection (DPBS). At each time point the blank group, which was subsequently injected with DPBS and FASW (D-V-), was regarded as the control, which is the base line of mRNA transcription. The transglutaminase-1 (TGase-1) expression of three different treatment groups is shown (**Fig. 5.2**).

At 1.5, 6, 9 hpp, there are significant DnaK-*Vibrio* interactions on TGase-1 expression (two-way ANOVA, $p < 0.05$). From 0 to 6 hpp, *Vibrio* is the main factor, which strongly elicit TGase-1 expression ($p < 0.0001$). DnaK doesn't affect TGase-1 expression. But the DnaK presence significantly attenuated the influence of *Vibrio* on TGase-1 expression at 1.5 and 6 hpp (two-way ANOVA, $p < 0.05$).

3.2 Prophenoloxidase (proPO-2) mRNA transcription

Prophenoloxidase (proPO) is a central enzyme in the prophenoloxidase activating system (proPO-AS). In *L. vannamei*, two proPOs genes have been identified. Contrary to the proPO-1 gene, the proPO-2 gene responds to DnaK via injection within 12 hours post injection (chapter 4). **Figure 5.3** shows proPO-2 expression of three treatment groups (D-V+, D+V-, D+V+), their proPO-2 expression is relative to the control group (D-V-), which is assumed as 1.000.

DnaK injection significantly up-regulated proPO-2 expression both at 1.5 and 9 hpp ($p < 0.001$). On the other hand, *Vibrio* elicited proPO-2 transcription to 4.6-fold at 9 hpp ($p < 0.0001$). At the same time point, DnaK-primed shrimps and then infected with *Vibrio* (D+V+), increased proPO-2 expression to 10-fold. However, there is no significant interaction between DnaK and *Vibrio*. The data indicate, at that time point, an additive effect from the two immune elicitors, DnaK and *Vibrio*. At 12 hpp, an antagonistic effect was observed on the proPO-2 expression from DnaK and *Vibrio* (two-way ANOVA, $p < 0.05$).

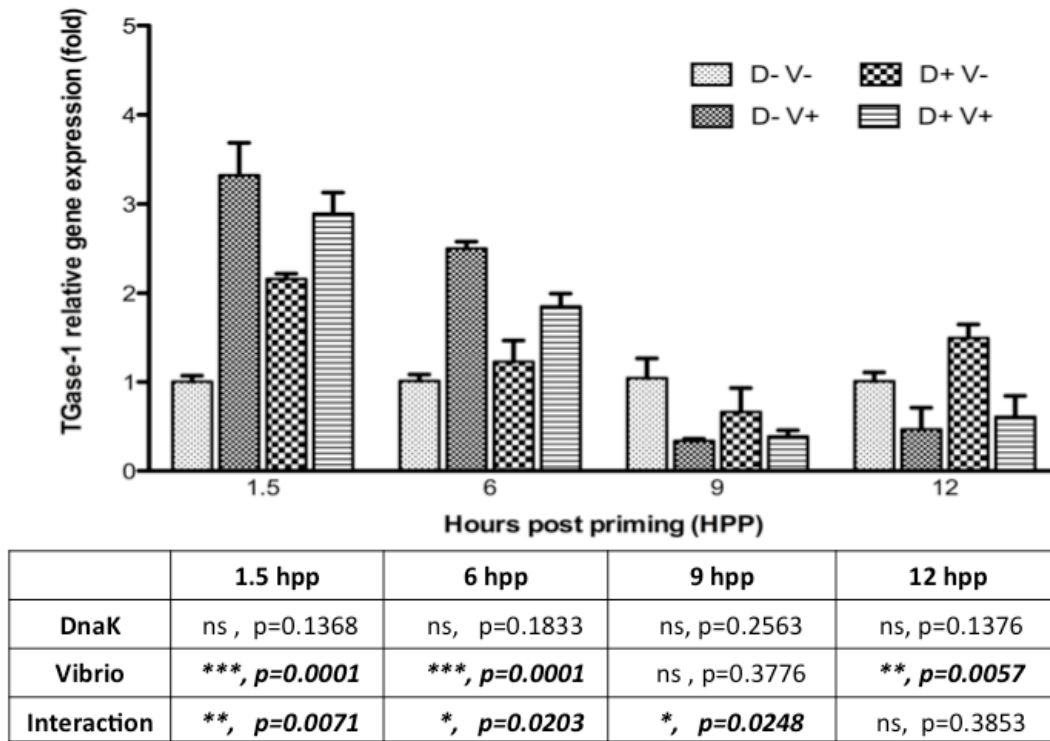


Figure 5.2. Expression of transglutaminase-1 (TGase-1) in non-lethal *Vibrio*-challenged *L.vannamei* primed with recombinant bacterial HSP70 (DnaK). The recombinant DnaK was injected in *L. vannamei* intramuscularly at a dose of 0.05 µg/shrimp. 1 hour after DnaK priming, *L. vannamei* were challenged with a non-lethal dose of *V. campbellii* (10^5 CFU). Hemocytes from 3 shrimps were sampled at 1.5, 6, 9 and 12 hours post DnaK priming (hpp). mRNA expression was analyzed by quantitative real time PCR (qRT-PCR). Bars indicate standard errors. Statistics: two-way ANOVA was applied to analysis TGase-1 expression affected by (a) DnaK priming (b) non-lethal challenge of *V. campbellii* and (c) their interactions, followed by Bonferroni post-test analysis. Significant differences at corresponding time point are indicated by ***($p < 0.001$), **($p < 0.01$), *($p < 0.05$).

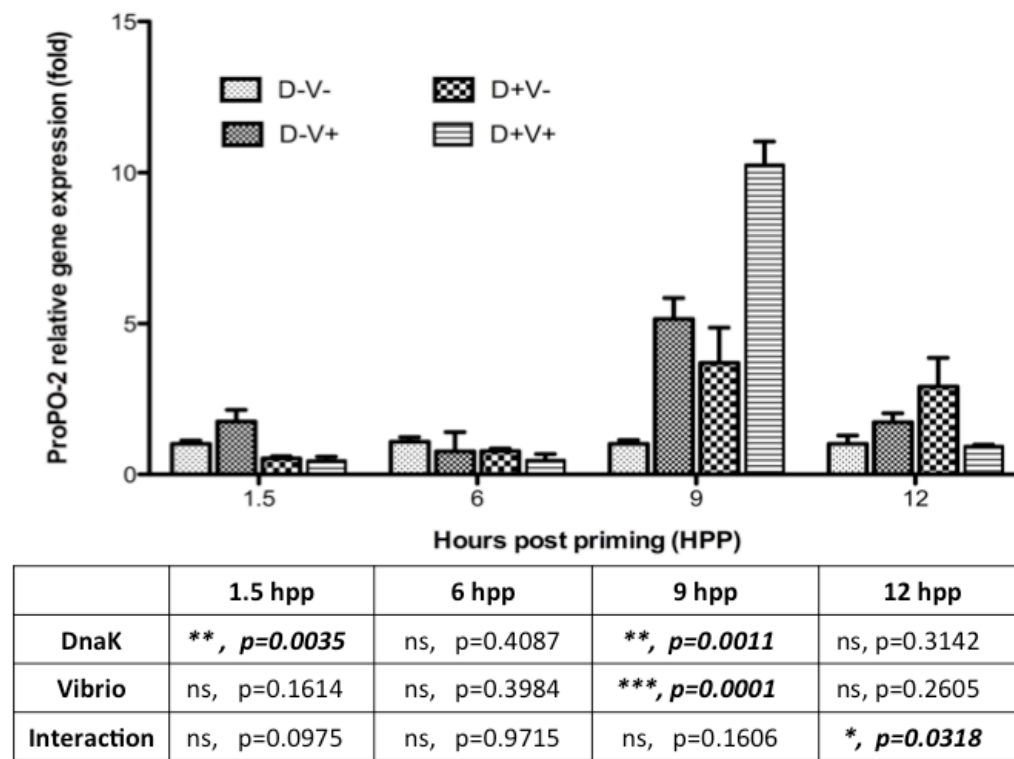


Figure 5.3. Expression of prophenoloxidase-2 (proPO-2) in non-lethal *Vibrio*-challenged *L. vannamei* primed with recombinant bacterial HSP70 (DnaK). The recombinant DnaK was injected in *L. vannamei* intramuscularly at a dose of 0.05 µg/shrimp. 1 hour after DnaK priming, *L. vannamei* were challenged with a non-lethal dose of *V. campbellii* (10^5 CFU). Hemocytes from 3 shrimps were sampled at 1.5, 6, 9 and 12 hours post DnaK priming (hpp). mRNA expression was analyzed by quantitative real time PCR. Bars indicate standard errors. Statistics: two-way ANOVA was applied to analyse proPO-2 expression affected by (a) DnaK priming (b) non-lethal challenge of *V. campbellii* and (c) their interactions, followed by Bonferroni post-test analysis. Significant differences at corresponding time points are indicated by ***($p<0.001$), **($p<0.01$), *($p<0.05$).

3.3 Endogenous *L.vannamei* HSP70 (lvHSP70) gene transcription level

The endogenous lvHSP70 transcription was monitored in our study as well (**Fig. 5.4**). Interestingly, all the treatments were not able to affect the lvHSP70 expression at most of the time point, except the combined treatment (D+V+) at 6 hpp. The lvHSP70 expression of the combination treatment (D+V+) was up-regulated 6.6-fold ($P<0.001$) at 6 hpp. A statistically significant synergistic effect

from DnaK and *Vibrio* challenge was observed at 6 hpp (two-way ANOVA, $p=0.0011$).

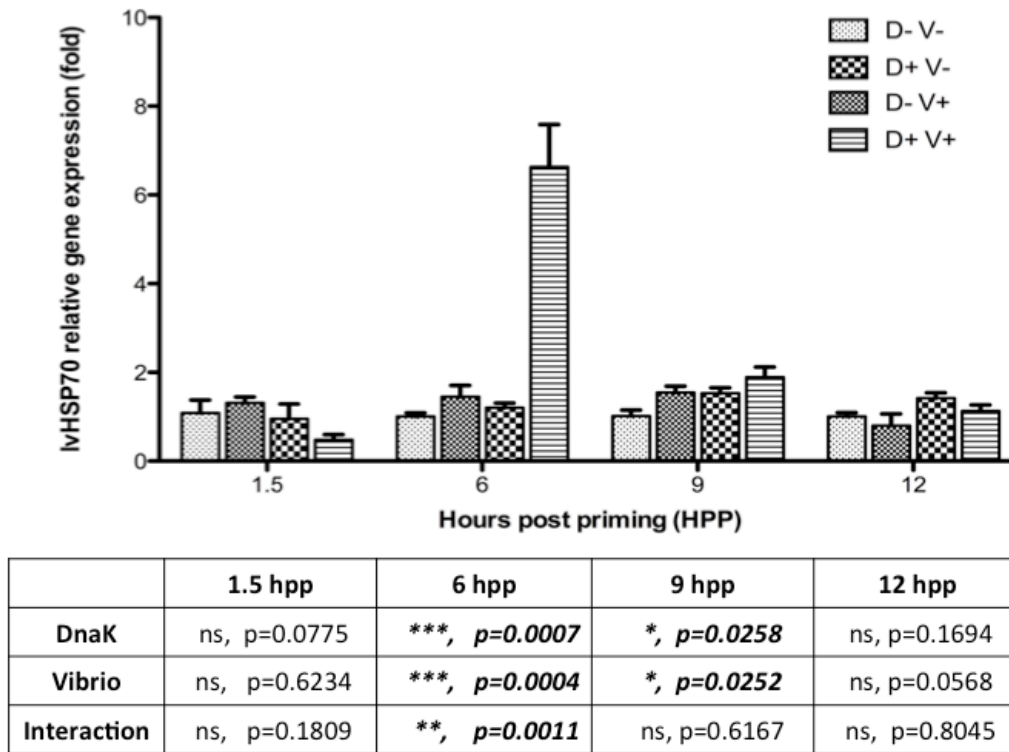


Figure 5.4. Expression of endogenous HSP70 (lvHSP70) in non-lethal *Vibrio*-challenged *L.vannamei* primed with recombinant bacterial HSP70 (DnaK). The recombinant DnaK was injected to *L. vannamei* intramuscularly at a dose of 0.05 $\mu\text{g}/\text{shrimp}$. 1 hour after DnaK priming, *L. vannamei* were challenged with a non-lethal dose of *V. campbellii* (10^5 CFU). Hemocytes from 3 shrimps were sampled at 1.5, 6, 9 and 12 hours post DnaK priming (hpp). mRNA expression was analyzed by quantitative real time PCR (qRT-PCR). Bars indicate standard errors. Statistics: two-way ANOVA was applied to analyse HSP70 expression affected by (a) DnaK priming (b) non-lethal challenge of *V. campbellii* and (c) their interactions, followed by Bonferroni post-test analysis. Significant differences at corresponding time points are indicated by ***($p<0.001$), **($p<0.01$), *($p<0.05$).

4. Discussion

In the pathogen-host interaction during bacterial infection, phagocytosis is a general and powerful weapon eliminating invading pathogenic bacteria. The phagosome acidification, oxidative burst, and phagosome fusion with lysosomes, stimulate bacterial heat shock protein expression and thus play an important role in helping bacteria to cope with a stressful environment. Among the bacterial HSPs families, DnaK is one of the best-characterized bacterial chaperones. Its role in protein folding is well studied. The extracellular HSP confer danger signals both in innate and adaptive immunity (Osterloh and Breloer, 2008). The HSPs produced by bacterial pathogens during infection have been reported to alert the host immune system and possibly results in increasing bacteria clearance (Stewart et al., 2001). Additionally, there are several reports suggesting a role for the bacterial chaperones (HSP60 and HSP70 family) at the cell surface, as adhesions for invading the host cell or in signaling the immune system (Pizarro-Cerdá and Cossart, 2006). In our study, the TGase, a key enzyme activating the clotting cascade and hemocytes aggregation in crustaceans (Maningas et al., 2008), was up-regulated by DnaK in the early hours (0-6 h) post injection. Furthermore, DnaK stimulated the central enzyme of the proPO cascade, proPO-2, 5.0-fold at 9 hpp. The up-regulation of two key immune-related genes suggested that DnaK plays an important role in *L. vannamei* immunity, and it most likely modulate the immune system in specific signal transaction pathways, interacting with clotting cascade and the proPO-activating system.

V. campbellii is widely distributed in the marine environment and pathogenic strains cause disease (also as luminescent vibriosis) in wild and cultured aquatic organisms including shrimp, finfish and molluscs, causing severe losses to the aquaculture sector (Austin and Zhang, 2006). The pathogenesis of *Vibrio* consists of entry of the pathogen, multiplication, avoidance of host defenses, causing damage and exit (Donnenberg, 2000). The virulence of *Vibrio* can be attributed to several factors, such as adhesion, production of extracellular polysaccharides and lytic enzymes, quorum-sensing, etc. (Ruwandeeepika et al., 2012). In this study, two immune-related gene, TGase-1 and proPO-2, were up-regulated by a non-lethal dose of *V. campbellii* (10^5 CFU/shrimp) in our study. It is consistent

with the observations that TGase-1 (Gene bank accession no. EF081004) and proPO-2 (Gene bank accession no. EU373096.1) transcription were elicited shortly (6 h, 12 h respectively) after a small amount of *Vibrio alginolyticus* (5×10^3 CFU/shrimp) was injected in *L. vannamei* (Yeh et al., 2009a; 2009b). Based on this and other studies, the proPO has been suggested critical for the defense against pathogenic infection in shrimps (Amparyup et al., 2009; Charoensapsri et al., 2011; 2009; Fagutao et al., 2009; Robert, 2003; Srivastava and Amato, 2001). This up-regulation of proPO-2 expression is also consistent with previous research in which the proPO gene was found to be up-regulated in crustaceans by potential immune stimulants or bacterial challenge (Arockiaraj et al., 2013; Hauton et al., 2007; Liu et al., 2013; Moseley, 2000.). The up-regulation of proPO generally coincided with a high level of immunity and resulted in higher survival (Hauton et al., 2007; Hsieh et al., 2013; Tobian et al., 2004; Wang et al., 2008). Thus, all evidences suggest that a non-lethal dose of *Vibrio* is an immune elicitor as well. On the other hand, in our study, endogenous HSP70 expression was significantly affected by this non-lethal dose of *V. campbellii* at 6 and 9 hpp. This result is similar to the finding of Zhou et al. (2010) that endogenous HSP70 expression is modulated within 12 h post *V. alginolyticus* injection. HSP70 is well known for its molecular chaperon and cytoprotective function (Pockley, 2003). Multiple approaches, for instance non-lethal heat shock (NLHS), or using chemical HSP70 induction applied to elevate endogenous HSP70 production and successfully help the aquatic animals (invertebrates) against *Vibrio* infection (Baruah, 2012; Sung et al., 2008). It indicates that the endogenous (lvHSP70) up-regulation is not only an indicator of *V. campbellii* invasion but also might help host to cope with this stressful context (*V. campbellii* challenge), probably through multi-functions of HSP70 in the immune modulation.

In this research, statistically significant interactions of DnaK and *V. campbellii* on the transcription of three immune-related genes were observed. When shrimp were primed with DnaK and then subsequently stimulated by a non-lethal dose of *V. campbellii*, the TGase-1 transcription was up-regulated immediately after *V. campbellii* injection but there is an antagonistic effect of the two immune elicitors (DnaK and *V. campbellii*). TGase is the central enzyme of

the clotting cascade in crustaceans. The clotting occurs through polymerization of a clotting protein in plasma and is catalyzed by a calcium ion dependent TGase (Hall et al., 1999; R. Wang et al., 2001). It is the first barrier of the host immune defense that traps the invading microbes (Z. Wang et al., 2010). It is very likely that the TGase response happens immediately after bacterial enter into the host. In that respect, **Fig 5.2** shows an overall downwards trend in TGase expression. The antagonistic effect, at the first 3 time points (1.5-9 hpp) might be explained by the hypothetical TGase expression kinetics shown in **Fig. 5.5**: a positive synergistic effect may appear before our first observation (dashed box; which is technically impossible to sample with the current overall protocol), allowing for a rapid elimination of *V. campbellii*, and reducing the need for increased expression at the later time points (1.5, 6 and 9 hours). The priming of TGase-1 by DnaK, resulting in a synergistic effect on TGase-1 expression upon *V. campbellii* challenge, would hence be a very early event. This interpretation would need to be substantiated by further experiments. In an alternative interpretation it might be that DnaK actually down regulates the early TGase-1 response (for unknown reasons), probably leaving a chance that injected *V. campbellii* is incompletely eliminated. To document further what is actually ongoing in the shrimp in these early hours post the double treatment (DnaK and *V. campbellii*), it would be advisable to also estimate the amount of circulating *Vibrio* in the hemolymph.

Shrimps pretreated with DnaK and subsequently treated with *V. campbellii* showed a strong synergistic effect on lvHSP70 expression ($p < 0.0001$, 6 hpi). The HSPs are regarded as “stress proteins” and are essential to cell survival (Jolesch et al., 2012). Bacterial infection is a stress condition to the host. Endogenous HSPs, especially HSP70, expression has been generally found as a marker in many infection studies (Ding et al., 2012; X. Wang et al., 2010). Therefore, the synergistic lvHSP70 expression is most likely the effect of damage (stress) caused by a *V. campbellii* infection. The latter might be caused by an incomplete elimination (at least relative to the other treatments) of injected *Vibrio* due to TGase-1 downregulation (see above) resulting in *Vibrio* regrowth. Again circulating *Vibrio* would need to be quantified to substantiate this interpretation.

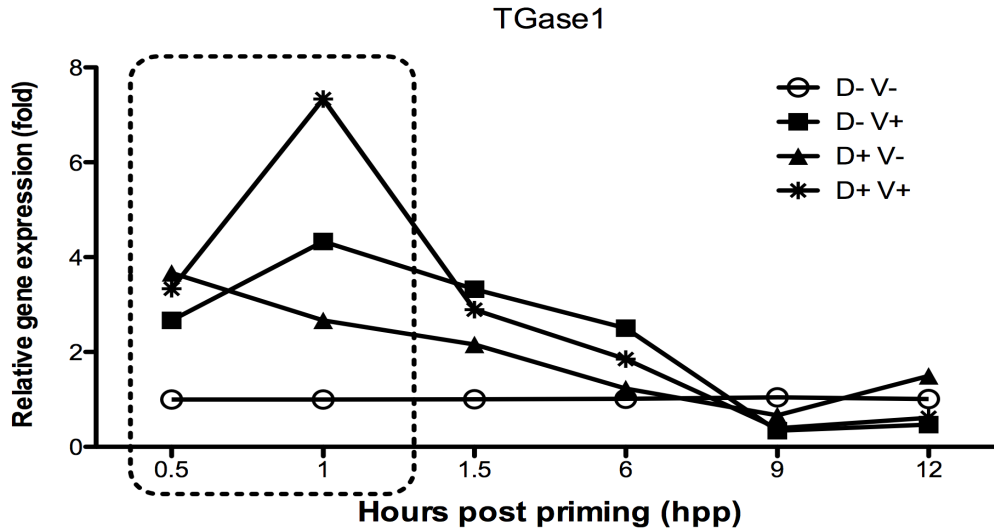


Figure 5.5 Hypothetical Transglutaminase-1 (TGase-1) expression kinetics post DnaK and *Vibrio* injection. The boxed area is hypothetical. The data outside the box are taken from Fig 5.2.

Comparing the expression dynamics of the three immune-related genes (TGase-1, proPO-2 and lvHSP70), it appears that the TGase-1 expression peaked the earliest (before 1.5 hpp), the second is lvHSP70 (6 hpp) and proPO-2 peak induction appeared 3 h later at 9 hpp. We assume that the chronological order of the three gene expression peaks may suggest a physical regulation order. The TGase is known as a clotting enzyme in crustaceans and proved to form a clot in order to trap pathogens. Therefore, it would respond as soon as the pathogen enters. However, we suppose not all the *Vibrio* would be entrapped and eliminated by the clotting cascade while part of *Vibrio* escape. Their proliferation may trigger lvHSP70, and result in the up-regulation of proPO transcription later on. The proPO-2 up-regulation peak happened 3 h after lvHSP70's synergistic increase. It is similar to the finding that in a feeding trial of DnaK overexpressing *E.coli* to *Artemia* and then challenged with *V. campbellii*, the proPO gene expression was significantly promoted (Baruah et al. 2011). It may suggest that the lvHSP70 up-regulated production induced by *Vibrio* elicited the proPO-2 transcription. This might require lvHSP70 relocalization, from intracellular to extracellular due to *Vibrio* induced cell damage/necrosis, a process that seems to be operational as proposed by Rozhkova et al (2010) that extracellular HSPs

could be internalized by interacting with cell surface receptors. However, that has not been documented in this study. Hence, this hypothesis still needs to be verified by further research.

Even though the invertebrate immune system is described as a non-specific innate immunity, increasing evidence suggest that hosts, pre-treated either with pathogen-derived molecules such as lipopolysaccharide (LPS) or non-lethal pathogen challenge, have better survival when subsequently exposed to a pathogen challenge (Sadd and Schmid-Hempel, 2006). This priming effect was also described as “adaptive innate immunity” (Moret and Siva-Jothy, 2003) or “trained immunity” (Netea et al., 2011) in invertebrate. Almost all of these mechanisms act in invertebrate immune systems and they represent the prerequisite for generating specific immunity. One possible mechanism for invertebrates generating specific immunity may be offered by the recent discovery of alternative splicing of the Down syndrome cell adhesion molecule gene (Dscam) (Dong et al., 2006; Watson et al., 2005). The Dscam was identified in *L. vannamei* and its isoform showed high response to natural pathogen *Vibrio harveyi* (Chiang et al., 2013 ; Chou et al., 2011). At the moment there is no evidence for a link between the high amount of isoforms formed by the Dscam genes in response to immunostimulants and the apparent synergistic (at least at some time points) regulation of gene expression for the 3 genes under investigation here.

In conclusion, we present for the first time *in vivo* evidence that bacterial DnaK can generate an altered and sometimes enhanced (priming) immune-related gene transcription when *L. vannamei* is subsequently infected with *V. campbellii*.

CHAPTER 6

BACTERIAL HSP70 (DNAK) PRIMES THE IMMUNE
SYSTEM OF *LITOPENAEUS VANNAMEI* AGAINST WHITE
SPOT SYNDROME VIRUS (WSSV)

Abstract

White Spot Syndrome Virus (WSSV) is a notorious viral pathogen, and it has caused huge loss in shrimp farming. Lots of efforts have been made to eliminate the mortality of this viral infection. Among all the solutions, enhancing immune resistance of the host is a promising approach to control WSSV infection. It is generally accepted that shrimps, like other invertebrates, solely rely on their innate immunity, which is lacking specific immune memory. But there is increasing evidence that utilizing virus-derived components or immunostimulants, enhance shrimp immunity moderating subsequent effects of WSSV infection. Bacterial HSP70 DnaK, which is proven to be highly immunodominant in *Litopenaeus vannamei*, was used to prime the shrimp immune system, ahead of a challenge with the WSSV-Viet strain which displays a reduced virulence relative to other strains. Transcriptions of two immune related genes, transglutaminase-1 (TGase-1) and prophenoloxidase-2 (proPO-2) were quantified within 12 h post DnaK priming (hpp). Strong synergistic effects, induced by both DnaK and WSSV (D+W+), on TGase-1 and proPO-2 were exhibited at 1.5 hpp and 9 hpp respectively ($p < 0.05$). These results indicate that the application of bacterial HSP70 homologue DnaK could be considered as part of an overall strategy to fight WSSV in *L. vannamei*.

Introduction

White spot syndrome virus (WSSV) is one of the most dangerous pathogens in aquaculture. Since it was first reported in East Asia in 1992, WSSV became the most severe epidemic viral disease to the shrimp culture industry worldwide, resulting in tremendous economic losses. WSSV belongs to the genus Whispovirus under a new virus family of Nimavirida (Vlak, 2002). It is an enveloped virus with a large circular dsDNA about 300 kbp (Tsai et al., 2004; Van Hulten et al., 2001; Yang et al., 2001). This virus can infect many crustaceans causing high mortality and can be transmitted from species to another species (Kanchanaphum et al., 1998; Peng et al., 2001). It is generally considered that shrimp do not have a specific or adaptive immunity. They are thought to rely on innate (non-specific) immunity for internal defense against pathogens. Nevertheless, recent research reported an “enhanced immunity” or a “quasi immune response” of shrimps, indicating that special immune memory might exist in shrimps (Venegas et al., 2000). Since then, many studies have been done to discover a “vaccine” against WSSV by delivering inactivated virus, WSSV envelope proteins, recombinant DNA harboring WSSV protein gene into shrimp (Caipang et al., 2008; Ning et al., 2009; Rajesh Kumar et al., 2008).

Heat shock proteins (HSPs) are ubiquitous chaperon proteins from prokaryotes to eukaryotes (Srivastava, 2002). Physiological stress, such as infection, inflammation, exposure of the cell to toxins, promotes synthesis of intracellular heat shock proteins (Hsps). Therefore, HSPs are often referred to as stress proteins or molecular chaperones, and they influence the synthesis, structure, localization, and function of other cell proteins (Parsell and Lindquist 1993). The heat shock protein 70 (HSP70) family is the best studied HSPs family and it has been suggested that extracellular HSP70 molecules are “danger signals” that trigger serial immune regulation. In view of its immune functions, HSP70 is implicated in protection against pathogenic microbes on economically important organisms in aquaculture such as *Penaeus monodon* (la Vega et al., 2006) and *Artemia franciscana* (Sung et al., 2009b), perhaps by stimulation of the immune response.

In this study, a recombinant HSP70 homologue DnaK was intramuscularly injected into shrimps as immunostimulant (priming), ahead of a challenge by a

less virulent Vietnam WSSV strain (WSSV-Viet, 1 hour later). The dynamics of the expression of two important immune-related genes, prophenoloxidase-2 (proPO-2) and transglutaminase-1 (TGase-1), were monitored via quantitative real time PCR (qRT-PCR) allowing to study the effect of DnaK, WSSV and their putative interactions.

2. Materials and Methods

2.1 Recombinant DnaK production*

The *E.coli* strain E_{native} that expresses DnaK with a hexahistidine-tag was stored in 40% glycerol at -80°C. These bacteria were grown at 37°C for 24 h on Luria-Bertani (LB) agar and then to log phase (OD_{600} 0.4-0.6), in LB broth on a rotary shaker at 37°C. Overproduction of DnaK was induced by adding L-arabinose (0.5 mg ml⁻¹) at 37°C for 4 hours. After induction, the bacteria were transferred to sterile tubes, centrifuged at 2200g for 15 min, suspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (no calcium, no magnesium, Gibco®, Invitrogen). Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in a mini beadsbeater (Biospec, USA) and subsequently centrifuged at 2200 g for 1 min at 4°C. The supernatant protein was taken for DnaK isolation.

For DnaK isolation, Dynabeads® (Dynabeads® His-Tag Isolation & Pull down, Invitrogen) were used according to the recommended protocol. Briefly, cell lysate was incubated with Dynabeads® for 10 minutes. The Dynabeads® were washed with fresh DPBS 4 times. Finally the DnaK was eluted into elution buffer from the Dynabeads®. The elution buffer was exchanged to DPBS using the Amicon® Ultra Centrifugal Filter (Millipore) according to the manual. The isolated DnaK was stored at -80°C as aliquots for future application. The DnaK analysis is presented in appendix I.

2.2 Viral stock

The WSSV strain (WSSV-Viet) used in this study originated from diseased *Penaeus monodon* from Vietnam. This WSSV-Viet was studied before and was

* Repetitive description of previous chapter

shown to be less virulent relative to two other isolates from Thailand (Rahman et al., 2008). It did not produce any mortality within the first 36 hours post injection. Mortality increased to 100% in 9 days (Rahman et al., 2008). The original WSSV isolate was passaged once in crayfish (*Cherax quadricarinatus*). Crayfish gill suspension containing WSSV was received from Research Institute for Aquaculture No.2, Vietnam. Crayfish gill homogenates containing WSSV-Viet were passaged in specific-pathogen free (SPF) *L. vannamei* to produce inocula and determine infectious titers as described previously (Escobedo-Bonilla et al., 2005). Shrimp infectious dose 50% endpoint (SID₅₀) ml⁻¹ titers was 10^{5.8}. Inocula were stored at -70°C and dilutions used to challenge *L. vannamei* were prepared in sterile ice-cold phosphate-buffered saline (DPBS). A dose of 30 SID₅₀ was prepared in a volume of 50 µl by diluting the stock with phosphate-buffered saline (DPBS). WSSV inoculum (50 µl) was injected into muscle at the junction between the 3rd and 4th abdominal segments. As a control inoculum (mock), DPBS alone was used.

2.3 Experimental animals

Specific-pathogen-free (SPF) *L. vannamei* were imported from Shrimp Improvement Systems LLC (Florida, USA). Animals were tested by PCR for WSSV, TSV, IHHNV, YHV, IMNV, and NHP using OIE approved methodologies and primers. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL8–12). They were kept in a recirculation system at a water temperature of 28–29°C, 34 g l⁻¹ salinity and pH of 7.8–8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After 1 week their diet was shifted to A2 monodon high-performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Shrimps for experimental purpose were housed individually in covered 10 L aquaria, filled with Instant Ocean® seawater prepared with distilled water at a salinity of 35 g l⁻¹, provided with constant aeration and maintained at 27±1°C by air heaters. Shrimps were acclimated under the latter conditions for 3 days before any subsequent treatment. Feeding was skipped for 24 h prior to the injection.

2.4 Priming immune system of *L. vannamei* with DnaK and WSSV challenge

As shown in **Fig. 6.1**, 0.05 µg recombinant DnaK in 100 µl DPBS was injected intramuscularly into *L. vannamei* as a priming treatment. The DPBS was used as blank. One hour later, a standard viral challenge was performed via injection as described before (Phuoc et al., 2008). The hemolymph was collected at 5 time points post DnaK priming, 1.5, 3, 6, 9 and 12 hours post priming (hpp). Three animals from each treatment were sacrificed at each time point.

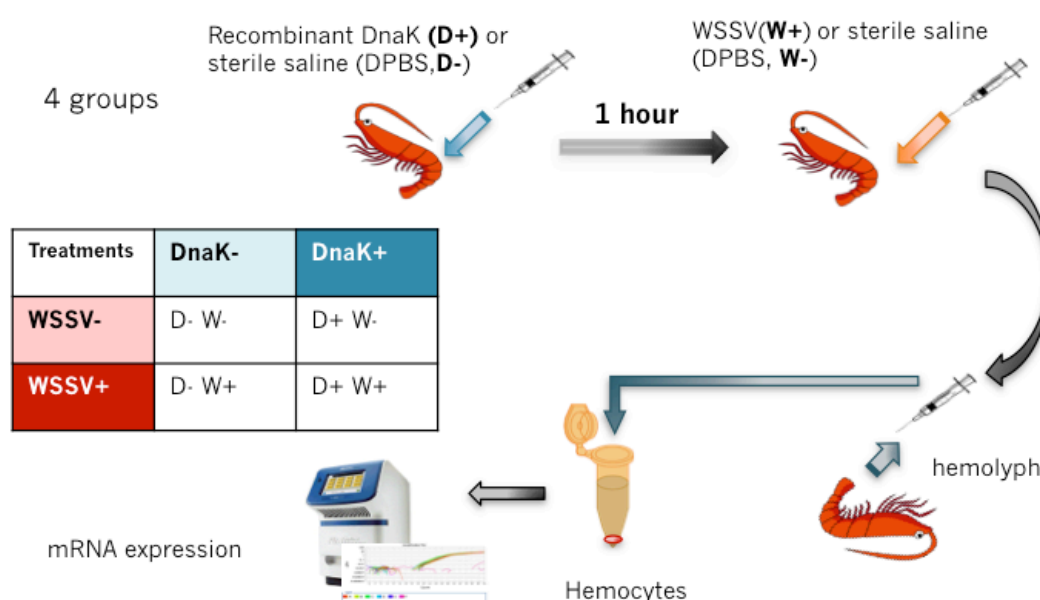


Figure 6.1. A schematic diagram of experimental design. Shrimps (*L. vannamei*) were assigned randomly and injected with either recombinant DnaK (0.05 µg, D+) or sterile saline (DPBS, D-); 1 h post DnaK injection (priming), the WSSV-Viet (30 SID₅₀, W+) or sterile saline (DPBS, W-) were injected. Hemocytes were sampled 1.5, 3, 6, 9, 12 hours post DnaK priming (hpp). At each time point, 3 shrimps from each treatment were sacrificed. Two immune-related genes transcription (proPO-2, TGase-1) were analyzed via qRT-PCR. Each cDNA sample was analyzed in two replicates.

2.5 Total RNA isolation and cDNA synthesis *

Hemolymph (200 µl) was collected from shrimp using a 1 ml syringe with 20G needle containing 300 µl of precooled anticoagulant (450 mM sodium chloride, 100 mM glucose, 30 mM tri-sodium citrate, 26 mM citric acid, 10 mM

EDTA, pH 5.4). After centrifugation of 250 g at 4°C for 5 minutes, the hemocytes pellets were quick frozen in liquid nitrogen and then preserved at -80°C for RNA extraction.

Total RNA was extracted from hemocytes using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Fermentas) to remove genomic DNA contamination, after which the RNA was quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was confirmed by electrophoresis. First strand cDNA was synthesized from 0.5 µg total RNA using the RevertAid™ 6H minus First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions.

2.6 Quantitative real-time PCR (qRT-PCR) analysis*

Two immune related genes, prophenoloxidase-2 and, transglutaminase-1, mRNA expression in *L. vannamei* was analyzed by qRT-PCR using a pair of specific primers designed on the basis of several *L. vannamei* cDNA sequence (**Table 6.1, GeneBank accession no.**). Real-time PCR was performed in an ABI StepOne Real Time System thermal cycler (Applied Biosystem) in a total volume of 20 µl containing 10 µl of 2× SYBR Green qRT-PCR Master mix (Applied Biosystems), 0.5 µl (each) forward/reverse primers (10 µM), 5 µl of cDNA template and 4 µl nuclease-free water (Sigma). The thermal cycle parameters used for the real-time amplification were: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 58°C for 30 s and elongation at 60°C for 30 s. Dissociation curve analysis in the real-time PCR was performed for each gene to check for the amplification of untargeted fragments. Data acquisition was performed with the StepOne software (v 2.2.2, Applied Biosystem) at the end of each elongation step.

* Repetitive description of previous chapter

Table 6.1 Gene bank accession No. and primer sequences used in qRT-PCR.

Gene	Gene Accession No.	Bank	Primers
Transglutaminase-1 (TGase-1)	EF081004.1		F: GAGCTTCAAGATCGAGGATCGA R: GCTGGTGTTTCGTAGCGGTTATC
Prophenoloxidase-2 (proPO-2)	EU373096.1		F: CTCAGCGTGAACCTCGCCTTA R: CCTGCTCAGTGTACGGTCT
β -actin	AF300705.2		F: GATGTGTGACGACGAAGTAG R: GATACCTCGCTTGCTCTGG

2.7 Real-time PCR data analysis*

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 0.5 μ g of RNA isolated from hemocytes samples. Serial dilutions of cDNA were amplified by real-time PCR using gene specific primers. ΔC_T (average C_T value of target – average C_T value of β -actin) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 1 for all of the target genes. Therefore, the amplification efficiency of reference and the target genes is equal. The expression of the target genes was normalized to the endogenous control β -actin (RNA polymerase A subunit) by calculating ΔC_T (Livak and Schmittgen, 2001):

$$\Delta C_T = C_{T, \text{target}} - C_{T, \beta\text{-actin}}$$

and expressed relative to control group (calibrator) by calculating $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T - \Delta C_{T, \text{calibrator}}$$

The relative expression was then calculated as

$$\text{Relative expression} = 2^{\Delta\Delta C_T}$$

2.8 Statistics

At each time point, data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test using statistic software GraphPad Prism Programs (GraphPad Prism, San Diego, CA) to determine significance. The

* Repetitive description of previous chapter

effect from two factors (DnaK, WSSV) and their interaction were evaluated. Values were considered significantly different if $p < 0.05$.

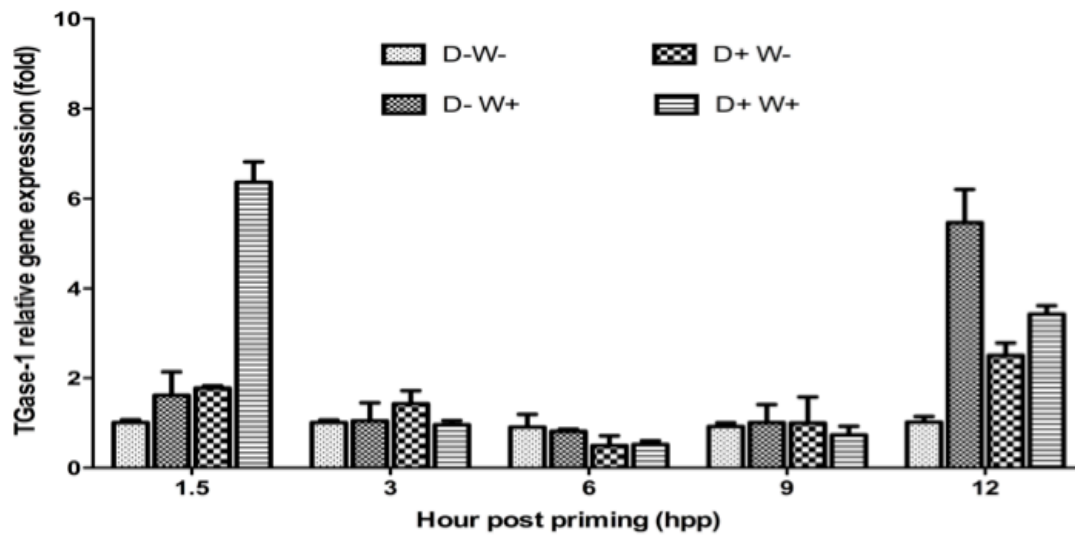
3. Results

3.1 Transglutaminase-1 (TGase-1) mRNA expression

During 12 hours post DnaK priming, the dynamics of TGase-1 transcription of four groups was monitored (**Fig. 6.2**). The group, which was subsequently injected twice DPBS (D-W-) was regarded as control and its TGase-1 expression level was assumed as 1.000. At two time points, significant up-regulation of TGase-1 transcription was observed (1.5 hpp and 12 hpp respectively). At 1.5 hpp, not only a significant modulating effect of two individual factors (DnaK, WSSV) was observed, but also a significant synergistic effect was observed (D+W+)(two-way ANOVA, $p < 0.0001$). At 12 hpp, it is showed that DnaK did not affect TGase-1 expression, while there is a significant interaction between DnaK and WSSV with DnaK significantly attenuating the TGase-1 response against WSSV. (two-way ANOVA, $p < 0.001$).

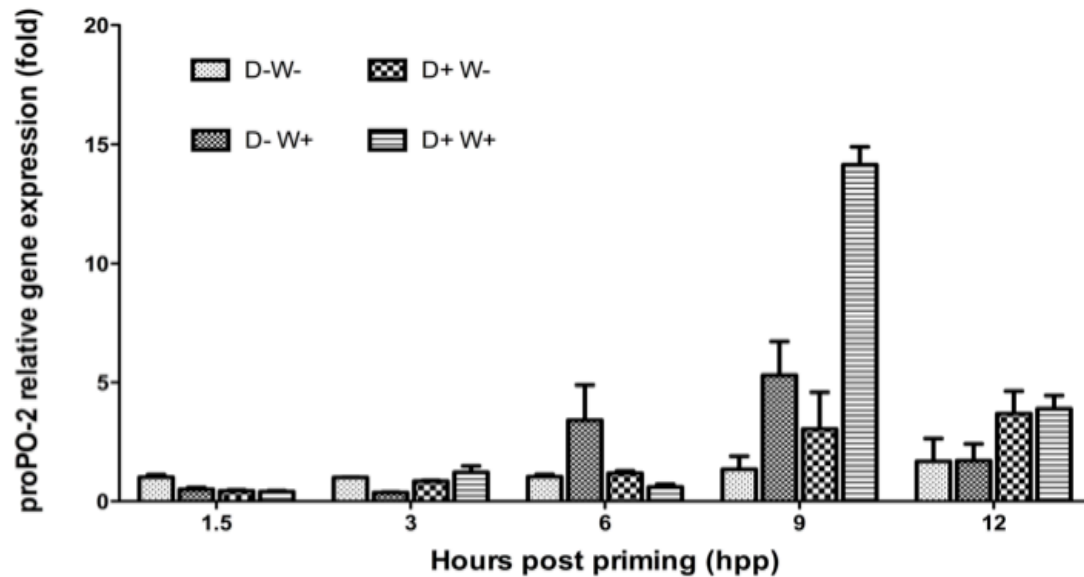
3.2 Prophenoloxidase-2 (proPO-2) mRNA expression

During 12 hours post DnaK priming, DnaK, WSSV and their combination affected proPO-2 expression (**Fig. 6.3**). ProPO-2 expression of all treatments increased dramatically after 6 hpp. At 9 hpp, both DnaK and WSSV up-regulated proPO-2 transcription. Meanwhile, there is a significant synergistic effect produced by both DnaK and WSSV (D+W+, two-way ANOVA, $p < 0.05$).



	1.5 hpp	3 hpp	6 hpp	9 hpp	12 hpp
DnaK	*** <i>p</i> =0.0005	ns	ns	ns	ns
WSSV	*** <i>P</i> <0.0001	ns	ns	ns	*** <i>p</i> =0.0002
Interac- tion	*** <i>P</i> <0.0001	ns	ns	ns	** <i>p</i> =0.0027

Figure 6.2 Expression of transglutaminase-1 (TGase-1) mRNA in WSSV-challenged *L. vannamei* pre-injected with recombinant bacterial HSP70 (DnaK). The recombinant DnaK was injected into *L. vannamei* intramuscularly at a dose of 0.05 µg/shrimp. 1 hour after DnaK priming, *L.vannamei* were challenged with WSSV-Viet (30 SID₅₀). Hemocytes from 3 shrimps were sampled at 1.5, 6, 9 and 12 hours post DnaK priming (hpp). mRNA expression was analyzed by quantitative real time PCR. Bars indicate standard error. Statistics: two-way ANOVA was applied to analysis TGase-1 expression affected by (1) DnaK priming (2) challenge of WSSV-Viet and (3) their interactions, followed by Bonferroni post-test analysis. Significant differences at corresponding time points are indicated by ***(*p*<0.001), **(*p*<0.01), *(*p*<0.05).



	1.5 hpp	3 hpp	6 hpp	9 hpp	12 hpp
DnaK	** <i>p</i> =0.0016	* <i>p</i> =0.0296	ns	** <i>p</i> =0.0018	ns
WSSV	** <i>p</i> =0.0051	ns	ns	*** <i>p</i> =0.0002	* <i>p</i> =0.0321
Interac- -tion	* <i>p</i> =0.0112	** <i>p</i> =0.0049	ns	* <i>p</i> =0.0144	ns

Figure 6.3 Expression of prophenoloxidase-2 (proPO-2) mRNA in WSSV-challenged *L.vannamei* pre-injected with recombinant bacterial HSP70 (DnaK). The recombinant DnaK was injected to *L.vannamei* intramuscularly at a dose of 0.05 µg/shrimp. 1 hour after DnaK priming, *L.vannamei* were challenged with a WSSV-Viet strain (30 SID₅₀). Hemocytes from 3 shrimps were sampled at 1.5, 6, 9 and 12 hours post DnaK priming (hpp). mRNA expression was analyzed by quantitative real time PCR. Bars indicate standard error. Statistics: two-way ANOVA was applied to analysis proPO-2 expression affected by (1) DnaK priming (2) challenge of WSSV-Viet and (3) their interactions, followed by Bonferroni post-test analysis. Significant differences at corresponding time points are indicated by ***(*p*<0.001), **(*p*<0.01), *(*p*<0.05).

4. Discussion

HSP70 family members are crucial to the folding and assembly of other cellular proteins, and they are also involved in the regulation of kinetic partitioning between folding, translocation and aggregation within the cell. They

also have a wider role in the regulation to the immune system, apoptosis and various facets of the inflammatory process (Roberts et al., 2010). Previously, it was demonstrated that the endogenous HSP70 and exogenous HSP70 (DnaK) shared the same protective efficiency to *Artemia* against *Vibrio campbellii* by oral feeding of recombinant HSP70 overexpressed in *E.coli* (Baruah et al., 2010; Sung et al., 2009a). In this study, immune response were monitored after an exposure (1 hour) to DnaK, followed or not by a challenge to a WSSV-Viet strain. The DnaK and WSSV exposure resulted in significantly strong synergistic effects on both proPO-2 and TGase-1 transcription at different time points.

White Spot Syndrome Virus (WSSV) is a notorious pathogenic virus, which causes White Spot Syndrome (WSS) and results in 90-100% mortality of shrimp (Flegel et al., 2008). Due to the severe impact of WSSV on shrimp farming, plenty of research has been done to unveil the mechanism of WSSV pathogenesis in shrimps and to find prophylactic or therapeutic solutions (Chen et al., 2008; Masuda et al., 2012; Rajesh Kumar et al., 2008; Srivastava, 2002). The proPO-activating system has been well studied in crustaceans. It mainly consists of several genes, such as serine proteinase and its inhibitors, prophenoloxidase-activating enzyme (PPA), proPO and its activated form phenoloxidase (PO) (Amparyup et al., 2013). The activation of the proPO-activating cascade is triggered by lipopolysaccharides (LPS), β -1,3-glucans or peptidoglycans (PG) (Söderhäll and Cerenius, 1998), which are the important cell-wall components of bacteria or fungi. The proPO-activating cascade participates in host defense, in arthropods by enhancing phagocytosis, initiating nodule or capsule formation, mediating coagulation and producing antibacterial/fungi substances (Cerenius and Söderhäll, 2004). Research indicated that there is no significant difference on the proPO transcription level and the enzyme activity of PO, between WSSV injected and Sham injected crayfish. Thus it was proposed that WSSV inhibited the proPO system upstream of phenoloxidase or the native substrate for the enzyme is simply consumed (Jiravanichpaisal et al., 2006). However, other different proPO gene expression kinetics was observed in shrimps. Li et.al (2012) showed that the proPO expression was up-regulated from 6 h post WSSV challenge in red swamp crayfish (*Procambarus clarkii*), which is consistent with our finding that the WSSV injection up-regulated proPO-2 expression after 6 h.

On the other hand, down-regulation of proPO-2 (GeneBank accession no. EF565469, 99% similarity to the proPO-2 measured in our research) induced by WSSV was recorded in *L. vannamei* (Ai et al., 2009). The mechanism behind the modulation of proPO expression in shrimp challenged with WSSV has not been fully elucidated. However a reactive component 5,6-dihydroxyindole (DHI), which is generated by PO, not only shows antibacterial and antifungal activities, but also exhibits strong toxicity against virus pathogen. It indicates that the proPO-activating system is also involved in the antiviral immune defense (Zhao et al., 2011). We think that the variable proPO expression dynamics, observed in the different studies, with WSSV challenged shrimps is caused by the variability in virulence of the WSSV isolates and the lack of the application of a standard challenge dosage. Therefore, in the research of WSSV-host interaction, the WSSV virulence and dosage should be carefully evaluated.

There is little evidence about the impact of WSSV challenge on TGase expression in shrimps. In crustaceans, the blood clotting process involves cross-linking aggregates of clotting proteins (CPs) that is catalyzed by a calcium-dependent transglutaminase (TGase) (Maningas et al., 2013). The TGase appears to be released from the hemocytes under foreign particle stimulus or tissue damage (Huang et al., 2004; Kopacek et al., 1993; R. Wang et al., 2001). Because invertebrates have an open circulatory system, TGase is a very important factor to ensure fast efficient wound healing and to prevent systematic infection (Loof et al., 2011). Recent research suggests that the TGase from *Drosophila* accumulate on microbial surfaces, leading to their entrapment in the clot (Z. Wang et al., 2010). Similar results were obtained in Kuruma shrimps (*Marsupenaeus japonicus*). TGase gene-silenced shrimps showed mortality when challenged with either White Spot Syndrome Virus (WSSV) or *Vibrio penaeicida* (Maningas et al., 2008). Additionally, in *Pacifastacus leniusculus*, knocking out the TGase gene led to morphological changes of hemocytes. It suggested that the TGase also has a critical function on blood cell formation and migration (Lin et al., 2008). In our study, the shrimps solely challenged with WSSV (D-W+) responded with an increased TGase-1 expression at 12 hpp after a quick increase at 1.5 hpp. It's very likely that the TGase-1 has multi-function in WSSV-host interaction.

In this research, there are significant interactions between DnaK and WSSV

on two immune related gene expressions. Significant synergistic effect were exhibited on TGase-1 and proPO-2 expression at 1.5 hpp and 9 hpp respectively ($p < 0.05$). Because of the absence of functional equivalents of T and B cells, invertebrate's response to infections solely rely on innate immune mechanisms (Lemaitre and Hoffmann, 2007). Recent research assumed that invertebrate innate immunity is completely devoid of memory traits. However, increasing evidences demonstrated an enhanced resistance to secondary infections induced by the first encounter with a pathogen (Roth et al., 2009; Sadd and Schmid-Hempel, 2006). This enhanced resistance is referred to be a “priming effect” in invertebrate immunity. Intriguingly, a group of studies showed that the highlighted resistance only existed when the host is re-infected by the same microorganisms (Pham et al., 2007). Moreover, the invertebrates pretreated with immunostimulant, such as lipopolysaccharide exhibited induced cross-protection as well (Moret and Siva-Jothy, 2003). There is some evidence to link hemocyte's phagocytosis to the enhanced immunity both in *Drosophila* (Pham et al., 2007) and the woodlouse, *Porcellio scaber* (Roth and Kurtz, 2009) when they are infected by bacteria. Even though the specific mechanism that underpins immune priming in invertebrate against viral infection is still not well understood, multiple approaches including pretreatment of the host with attenuated WSSV, WSSV components such as nucleotide, capsid proteins, immune stimulants or bacteria as “vaccine” against WSSV infection can improve the shrimp survival (Caipang et al., 2008; Khimmakthong et al., 2011; Kulkarni et al., 2013). In the crayfish, live attenuated bacteria provided a level of protection against WSSV (George et al., 2006). It is consistent with another research that transforming WSSV VP28 DNA into an attenuated *Salmonella* strain, subsequently fed to shrimps, resulted in protection against WSSV in crayfish (Ning et al., 2009). It suggested that bacteria or its components (for instance here DnaK) have the capacity to induce a general priming effect of the immune system of shrimp to fight against viral infection, for instance by modulating TGase or proPO. However, for reasons that are not totally clear yet, the dynamics among these 2 genes are different.

In conclusion, it is demonstrated here for the first time that priming the immune system of *L. vannamei* with a highly conserved and immunodominant

Chapter 6

bacterial component DnaK, augment TGase and proPO expression synergistically, be it with different dynamics.

CHAPTER 7

GENERAL DISCUSSION AND PERSPECTIVES

The increasing production aquaculture has suffered tremendous loss caused by infectious disease. Unlike terrestrial animals (mice, pigs and sheep), aquatic animals live in water, and hence their environment is much more crucial because the number of microorganisms in water is much higher. Diseases outbreak in aquaculture are complex, involving numerous effectors. It can be concluded that disease is an outcome of an imbalance of environment, host and microbial community (**Fig 7.1**). For aquatic animals, the environment and the microbial community are often referred to as abiotic and biotic stresses. Prevention of diseases and protection of aquatic animals against stress will play key roles in the successful growth of aquaculture. Therefore, prophylactic approaches such as vaccination and immunostimulants could be ideal tools for disease prevention in aquaculture. HSPs have been known as “stress proteins”, which are crucial parts for the stress trauma recovery. Additionally, the capacity of HSPs to regulate both innate and adaptive immunity makes them potential intriguing tools in immune therapeutic approach.

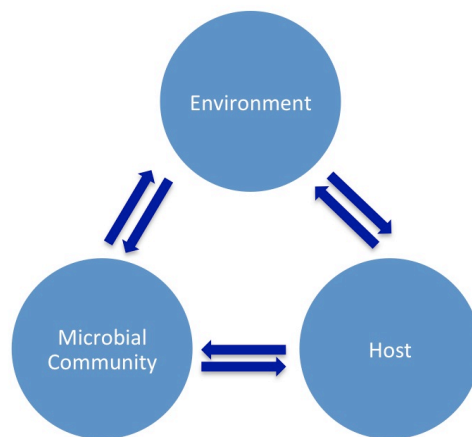


Figure 7.1 Host-pathogen interactions in a complex system

In this thesis, we aimed at investigating the immune effect of DnaK (bacterial HSP70) in *Litopenaeus vannamei* and its interaction with two dangerous microbial pathogens, *Vibrio campbellii* and White Spot Syndrome Virus (WSSV), via specific gene expression protocols. Firstly, the role of DnaK as an efficient

immune stimulator in *L. vannamei* was verified by comparing the dynamics of expression of two vital immune genes (transglutaminase and prophenoloxidase) as induced by either prokaryotic-derived recombinant DnaK or a chemically synthesized and microbial-contaminant-free DnaK peptide fragment (DnaK₄₄₂₋₄₉₁). Secondly, expression kinetics (during 12 h post DnaK injection, hpi) of several immune related genes, induced by two doses of DnaK was investigated. The prophenoloxidase-2 (proPO-2) and transglutaminase-1 (TGase-1) were selected as immune markers for subsequent study because of their significant response to DnaK. Finally, significant interaction between DnaK and immune gene expression as induced by the microbial pathogens *V. campbellii* or WSSV was observed at selected time points post the dual exposure to DnaK and a pathogen. We could conclude that the bacterial HSP70 homologue DnaK has the capacity of priming the immune system of *L. vannamei* responding to *Vibrio* and viral infection. The mechanism behind the phenomenon of DnaK priming in *L. vannamei* is still obscure. In this Chapter, we will discuss with “a birds eye perspective” the possible role of DnaK in the *L. vannamei* immune defense.

MICROBIAL HSP70 (DnaK) IN PATHOGEN VIRULENCE

Heat shock proteins (HSPs) are ubiquitous proteins existing in both prokaryotic and eukaryotic animals (Pockley, 2003). They are induced by a broad range of stress and perform cytoprotective functions. Therefore, the HSPs are also called “stress proteins” (Zhao and Jones, 2012). In pathogen-host interaction, when bacteria encounter stress due to phagosome acidification, oxidative burst, and phagosome fusion with lysosomes, the bacterial HSPs are induced in order to help bacteria cope with the stressful host environment, thereby aiding bacterial pathogenesis (Hosogi and Duncan, 2005).

In bacteria, the DnaK (HSP70) is involved in protein folding and is associated as well with survival under stress conditions (Genevaux et al., 2007). It is also implicated in bacterial virulence. Several studies have suggested that in *Brucella suis*, *Campylobacter jejuni*, and *Salmonella enterica*, deletion of DnaK results in compromised growth, in macrophages or inability of colonization (Konkel et al., 1998; Köhler et al., 1996; Takaya et al., 2004). Bacterial HSPs genes are elicited by the regulating alternate sigma factor or the transcription factor HspR (Heat

shock protein receptor). Manipulation of either of these factors affects heat shock response and also modulates bacterial virulence (Gophna and Ron, 2003; Kazmierczak et al., 2005). Furthermore, the HSP70 secretion ability is also related to virulence of pathogenic bacteria: Hoffman et al. (Hoffman and Garduno, 1999) suggested that when pathogenic bacteria intrudes the host, it secretes cell surface HSP70 to facilitate bacterial attachment to gastric epithelial cells, while non-pathogenic bacteria keep HSP70 intracellular. Pizarro and Cossart (2006) reviewed examples that suggest a role of bacterial chaperones at the cell surface, as adhesins for invading the host cell or in signaling the immune system. However, mere overexpression of microbial HSPs may not always confer virulence to bacteria. The work from Gupta et al. (2008) and Smith et al. (2003) showed that overexpression of DnaK resulted in enhanced clearance of bacteria in a mouse model. It suggests that increased DnaK production elicits the immune system early during infection possibly benefiting clearance of bacteria.

In pathogen-host interaction, the bacterial pathogen expresses a wide range of virulence factors that facilitate bacterial adhesion and invasion. On the other hand, the virulence factors also possess immune-modulatory effects. In higher vertebrate animals, the immunity to pathogenic bacteria can be divided into three stages (Fedele et al., 2013): 1) As soon as the bacteria enter into the host environment, it would be sensed by the immune cells such as macrophages and dendritic cells through pathogen recognition receptors (PRRs); 2) pathogen recognition initiate the host innate immune pathway and release cytokines with local pro- or anti-inflammatory effect; 3) subsequent adaptive immune response is ignited by dendritic cells (DC). In an invertebrate model, *Drosophila*, which possesses only an innate immunity, its immunity recognizes virulence factors of bacterium upon entry into host through two groups of receptors (Péan and Dionne, 2014): the first is pattern recognition receptors of the Toll pathway and immune deficiency (Imd) pathway, the second is phagocytic receptors and associated receptors including Scavenger receptors, Down syndrome cell adhesion molecule (Dscam), Peptidoglycan Recognition Proteins (PGRP)-domain containing receptors and so on. Until now, the Toll pathway, Imd pathway and Dscam have been identified in shrimp (Chou et al., 2009; Li and Xiang, 2013). It implicates that as a virulence factor, DnaK may trigger the immune system of

shrimps through the Toll pathway and the Imd pathway. Furthermore, Dscam is also a possible cell receptor which possibly recognizes DnaK.

All these literature evidences suggest that bacterial DnaK can be an important molecule in host-microbial interactions, justifying a further investigation of its role in the outcome of a host microbial interaction as verified in this thesis.

HSP70 AS IMMUNE REGULATOR IN THE HOST

Heat shock proteins are ubiquitous from eukaryotes and prokaryotes. They are both constitutively expressed during normal growth and profoundly induced by a range of cellular insults that cause protein damage within the intracellular environment. HSPs have been coined stress proteins. Thus, the expression of HSPs is a universal response to stress, which is fundamental to ensure survival. However, HSPs have also been found outside cells, which has been associated with significant immune modulatory functions, distinct from intracellular HSPs.

HSPs receptors and contaminants

Immune modulatory effects of HSPs have been reported recently, but there have been concerns that the observed effects might be because of associated compounds, which were present in the HSP preparation used or being bound to HSPs as contaminants (Tsan, 2004). In some *in vitro* immunological studies, the reported HSP-induced cytokine effects are very similar to those induced by pathogen associated molecule patterns (PAMPs), such as LPS, nucleotide, flagellin. As most bacterial recombinants are produced by *Escherichia coli* expressing HSP cDNA, the final preparation may be contaminated with bacterial products. Furthermore, TLR2, TLR4, CCR5 are reported to be cell surface receptors of the immune cells for HSP70 and HSP60 (Pockley et al., 2008), while LPS, etc. employ the same receptors as signal transducers. Thus, the microbial contaminants in HSPs preparation are a crucial issue in HSP immunological research.

Many studies have proven that HSP70 has immune regulatory properties when interacting with immune cells, such as monocytes, macrophages and dendritic cells (DCs)(Asea, 2002; Nace et al., 2012; Takaya et al., 2004). The extracellular HSP70 are regarded as a danger signal to immune cells. It induces

several immune reactions. However, the source of extracellular HSP70 has not been well identified. Basu et al. (Basu et al., 2000) showed that extracellular HSP70 assumed to be released after tissue necrosis, modulated the immune system. On the other hand, it was demonstrated that the release of HSP70 was not due to cell death but via a non-classical pathway, possibly involving lysosomal lipid rafts (Hightower and Guidon, 1989; Hunter-Lavin et al., 2004). Different scenarios have been proposed for the active export of HSP70 from the cytosol into the extracellular environment. There are studies suggesting that HSP70 is released through secretory-like granules (Evdonin et al., 2006) lysosome-endosome pathway or extracellular vesicles (ECVs) (De Maio, 2011).

The dual role as immune regulator

Although extracellular HSP70 has been suggested as a significant immune-regulator, in our study, a reverse dose-response of HSP70 was observed. The low dose of DnaK showed a stronger immune-stimulatory effect in *L. vannamei* and the high dose DnaK seems to attenuate the immune response. In a feeding trial of HSP70-overexpressing *E.coli* on *Artemia*, a significant deleterious outcome of an over-dosis was shown (Baruah et al., 2013). It suggests the extracellular HSP70 may have a capability of modulating immune response in two different directions (immune-stimulatory and immune-suppression). In higher vertebrate, the extracellular HSP70 no doubt are innate immune activators (Wallin et al., 2002). Asea et al. (2006) reported that exogenously added HSP70 can stimulate monocytes in a way of inducing an intracellular Ca^{2+} flux, activating nuclear factor kappa B (NF- κ B) and resulting in pro-inflammatory cytokine secretion via TLR2 and TLR4. Nevertheless, exogenous HSP70 as well can significantly suppress LPS-induced reactive oxygen species production in various myeloid cells and decrease Nitric oxide (NO) expression in macrophages, which is enhanced after LPS priming (Rozhkova et al., 2010). In Sea urchin, it was also reported that extracellular HSP70 may promote mitosis of dividing cells and suppress the immune cells reactivity (Browne et al., 2007).

The mechanisms underlying the dual immunoregulatory role of HSP70 is not yet fully understood. Cell-mediated immunity is a complex process, and there are several different stages at which HSP70 could regulate the qualitative nature of

this response. The observation that HSP70 have powerful pro-inflammatory effects on innate immune cells *in vitro* and immunosuppressive effects cannot be explained yet. However, it is noticeable that the activation of innate immune cells does not necessarily lead to a pro-inflammatory outcome because triggering of the large and diverse Toll Like Receptor (TLR) family can have both pro and anti-inflammatory consequences. Furthermore, activation of innate immunity can increase the expression and release of self-HSPs families, which in turn can activate other related immune regulatory networks that may have the capacity to control immune events. For example, the increase of self-HSP60 can stimulate specific immunoregulatory cell populations that have the capacity to control inflammatory events (Quintana and Cohen, 2011). These findings indicate the presence of a complex immunoregulatory HSP network *in vivo* that involves innate immune activation, release of self HSPs and the recruitment and activation of specific immune regulatory cell populations.

THE ROLE OF HSP70 IN APOPTOSIS

Stress or heat shock proteins (HSPs) are powerful chaperones whose expression is induced in response to a wide variety of physiological and environmental insults. These proteins have different functions depending on their intracellular or extracellular location. Intracellular HSPs have a protective function. They allow the cells to survive potentially lethal conditions. The cytoprotective functions of HSPs can also be explained by their anti-apoptotic properties (Joly et al., 2010): HSP70 can directly interact with different proteins of the tightly regulated programmed cell death machinery and thereby block the apoptotic process at distinct key points. The essential role of HSP70 in apoptosis has been confirmed by gene silencing studies. Intracellular HSP70 is a decisive negative regulator of apoptosis that can act: (1) at a pre-mitochondrial stage by inhibiting stress inducing signaling; (2) at the mitochondrial stage, by preventing mitochondrial membrane permeabilization, and (3) at the post-mitochondrial level by inhibiting caspase activation and DNA fragmentation. (Fig.7.2) (Joly et al., 2010).

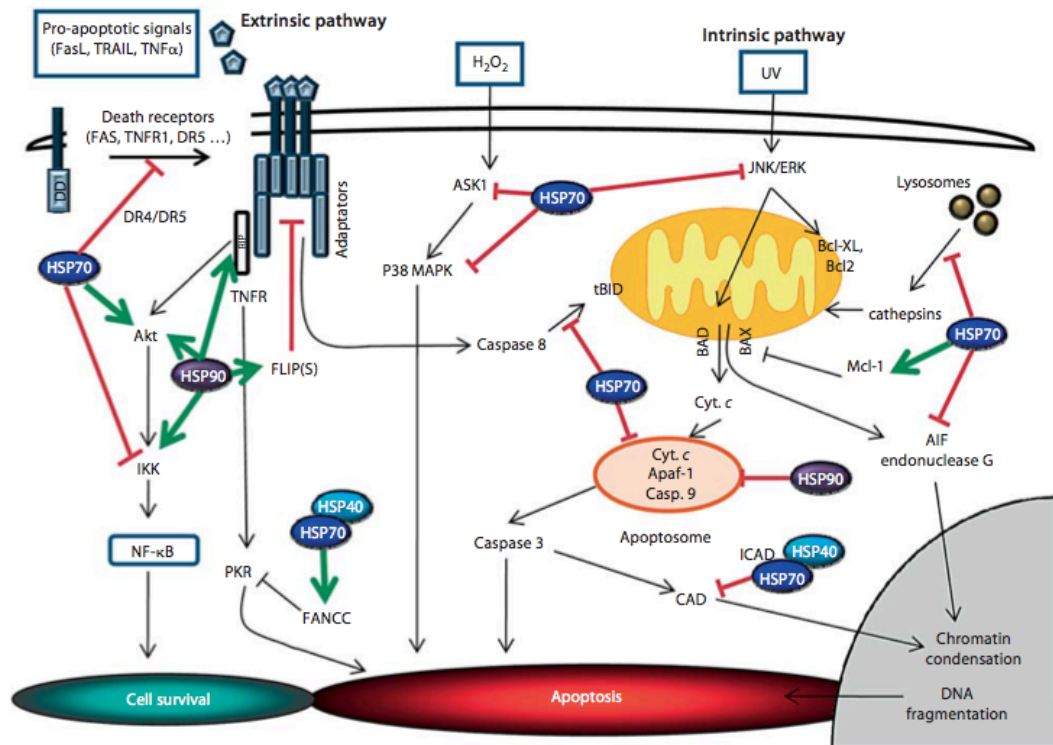


Figure 7.2 Schematic representation of intracellular HSP70 and HSP90 regulatory functions in the intrinsic and extrinsic pathways to death. HSPs can block the mitochondrial intrinsic pathway of apoptosis by interacting with key proteins at 3 levels: (1) upstream of the mitochondria, thereby modulating signaling pathways (HSP70 modulates the activation of stress-activated kinases such as Akt, JNK or ERK); (2) at the mitochondrial level, controlling the release of cytochrome c by interaction with BAX for instance; and (3) at the post-mitochondrial level, by blocking apoptosis by their interaction with APAF-1. HSPs can also block the extrinsic pathway at different levels. They can interact with the death receptors (DR4 and DR5), they can inhibit Bid cleavage and thus the activation of the mitochondria pathway and they can neutralize AIF and inhibit cathepsins. Arrows indicate increased activity while the barred ends suggest steps are blocked or reduced. (Joly et al., 2010)

A viral-induced, massive and uncontrolled apoptosis will lead to the death of the host (Flegel, 2009; Sahtout et al., 2001). From another point of view of bacterial virulence, *Vibrio* was initially proposed to kill cells by apoptosis (Broberg et al., 2011). Apoptosis has been shown to occur in *Epithelioma papulosum cyprini* (EPC) cells from a carp fish during infection with a *Vibrio*

alginolyticus strain (Zhao et al., 2010). In these scenarios, both viral- or bacterial-induced apoptosis are considered detrimental. The anti-apoptosis function of intracellular HSP70 may be the explanation for the results found in several studies that successfully applied heat treatment as a prophylactic/therapeutic approach against *Vibrio* and WSSV infection in crustaceans (Lin et al., 2011; Sung et al., 2007). There are ample evidences that a Non-lethal Heat Shock (NLHS) directly elevates HSP70 production in crustaceans (Baruah, 2012; Loc et al., 2013). Nevertheless, caution need to be taken while performing a NLHS on aquatic animals, as increasing the water temperature has a negative influence on the level of dissolved oxygen, the salinity and the concentration of toxic metabolites such as ammonia or nitrites, which is harmful for the aquatic animals. It is therefore important to find the balance between intracellular HSP70 induction and a deteriorated aquatic environment. While in this study, pretreating the host with exogenous HSP70 DnaK induced an elevated endogenous HSP70 transcription when a subsequent *Vibrio* infection was applied (chapter 5). Schmitt et al. (2006) illustrated that intracellular HSPs are the source of anti-apoptosis function. Thus, it appears that the recruitment of endogenous intracellular HSP70 might help to suppress pathogen-induced apoptosis.

BIOLOGICAL VARIABILITY AFFECTS THE DYNAMICS OF THE IMMUNE RESPONSE

In this research, two different specific pathogen free (SPF) shrimp resources were used: one batch is from the Sy-Aqua Siam Co. Ltd. Bangkok 10110, Thailand (Chapter 3&4); the batch came from Shrimp Improvement Systems (Florida, USA) (Chapter 5&6). Standardized protocols for materials (DnaK, salt solutions) and all handling procedures were always adopted. However, mRNA expression pattern of the same gene but from a different batch of shrimps are different (**Fig. 7.3**). For instance, the TGase-1 expression from batch I shrimps (Thailand) exhibited a sharp peak expression at 6 hour post DnaK injection in two independent experiments (DnaK and DnaK₄₄₂₋₄₉₁), while another batch of shrimps (USA) showed a completely different gene expression pattern. This phenomenon suggests that even in the same species, the genetic difference

and/or other unidentified factors may largely influence the dynamics of the immune response when treated with the same immunostimulant.

Diseases are a major constraint on the intensive shrimp farming. Conditions in production ponds favor disease development, and epidemics of several previously unreported diseases have occurred and caused severe losses. When elimination disease is difficult to perform, selective breeding for host resistance to the pathogen may be an attractive alternative for disease control. According to a theory proposed by Cock et al. (2009) host resistance is not a panacea and should only be considered when (a) the disease causes severe damage (b) there are no other existing simple cost effective control measures and (c) there is demonstrable genetic variation in resistance, and this is not coupled with an excessive level of negative associations with other desirable characteristics. Shrimp have only recently been domesticated and breeding for resistance only began in the mid 1990s; there is limited experience with shrimp breeding in particular and crustaceans in general. Consequently, the principles and concepts behind breeding programs are based largely on experiences with other species in aquaculture (Cock et al., 2009).

Various genetic markers can be used to identify particular individuals in a population. Microsatellites represent probably the most efficient and precise system that has been developed in several animal models for lineage, fitness, diversity and mapping. Immuno-markers constitute potent tools to select specific breeders. Current research is focused on lectins, hemolymph proteases and antibacterial capacities, phagocytosis-associated radicals and hemogram structures, to identify which criteria are best to characterize immune capacity.

At current stage, it is difficult to predict if the conclusions drawn across the different chapters are influenced by the different performance of the two groups of shrimp. It anyhow highlights that undetermined factors, probably genetic variation, might influence the outcome of the type of experiments performed in this study and that thorough standardization is required if firm conclusions are to be obtained.

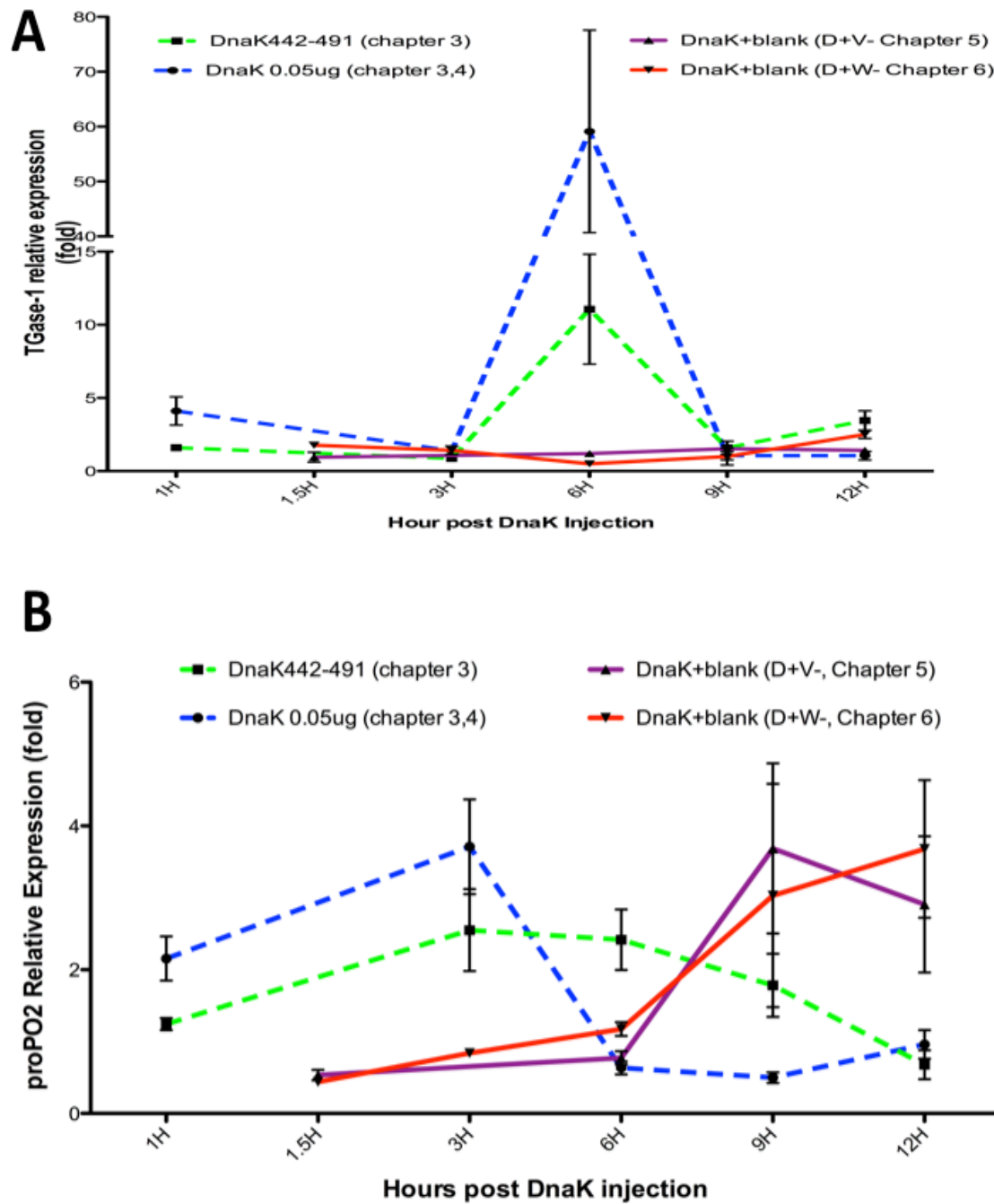


Figure 7.3 The dynamics of expression of two immune-related genes (A) Transglutaminase-1 (TGase-1) (B) prophenoloxidase-2 (proPO-2) from four independent experiments. The gene expression patterns within the same batch of shrimps are similar (blue and green dash lines), while another batch of shrimps exhibited a different pattern (purple and red lines).

CONCLUSION AND PERSPECTIVE

In our research, we verified the priming effect (as verified by specific gene expression) of a microbial HSP70 homologue DnaK in the immune system of *L. vannamei* against *V. campbellii* and WSSV infection. The immunostimulatory capability of bacterial HSP70 DnaK is a small piece of a major puzzle. The fate of prokaryotic HSP70 in shrimp and how this relates to immunostimulation and its capacity to inhibit pathogens, remain, among others, interesting topics for future research. In recent years, the knowledge of immune defense mechanism in shrimps is largely enriched. Several immune transaction pathways such as Toll pathway, IMD pathway and the JAK/STAT pathway have been discovered in shrimps (Li and Xiang, 2013). In addition, *via* the expressed sequence tag (EST) technique more immune components responding to bacterial and viral infection have been discovered in shrimps (Duan et al., 2013; Gross et al., 2001). This increased knowledge provides tools that could help us to better understand the immune function of prokaryotic HSP70 in shrimp.

In this research, an experimental DnaK administration route, intramuscular injection, was applied. The injection is the most direct administration route compared to feeding and immersion. However, it is impractical in aquaculture farming because of intensive labor work involved. Moreover, inappropriate animal manipulation would cause body damage and result in subsequent physiologic reaction in aquatic animals. For instance, cuticle damage is one of the causes of proPO activation so that melanization appears after shrimp traumatization (Amparyup et al., 2013). Feeding DnaK through heat shocked bacteria or DnaK overexpressing non-pathogenic bacteria are possible alternatives, which have been successfully applied in gnotobiotical *Artemia* resulting in *Vibrio* resistance (Baruah et al., 2010; Sung et al., 2009). HSPs are presumably degraded during digestion in the gut. It has been proven that the truncated DnaK overexpressed in *E.coli* almost equally protected as the full-length protein DnaK in gnotobiotic *Artemia* (K. Baruah et al., 2013). Also, it has been found that an HSP70 peptide consisting of amino acid residues 407-426 stimulates mammalian dendritic cells (Wang et al., 2005). In our research, a chemically synthesized DnaK fragment DnaK₄₄₂₋₄₉₁ also displayed an immunostimulatory effect. It will be interesting to verify if it is possible to obtain

immunostimulation in *L. vannamei* by feeding full-length protein or an epitope of it, either as such or encapsulated allowing proper delivery to the (uncharacterized) active site.

To summarize, the application through injection of bacterial DnaK has revealed that expression of genes known to be important immune components in *Vibrio* and viral resistance of *L. vannamei* is enhanced. In the future, more details about the immune function of prokaryotic HSPs in invertebrate immunity should be elucidated, knowledge that could become the basis for application in aquaculture. With the development of new tools, such as crustaceans immune cell cultures, and existing molecular methodologies, it will be possible to dissect the complex relationship between HSP's and components of the shrimp immune system.

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APPENDICES

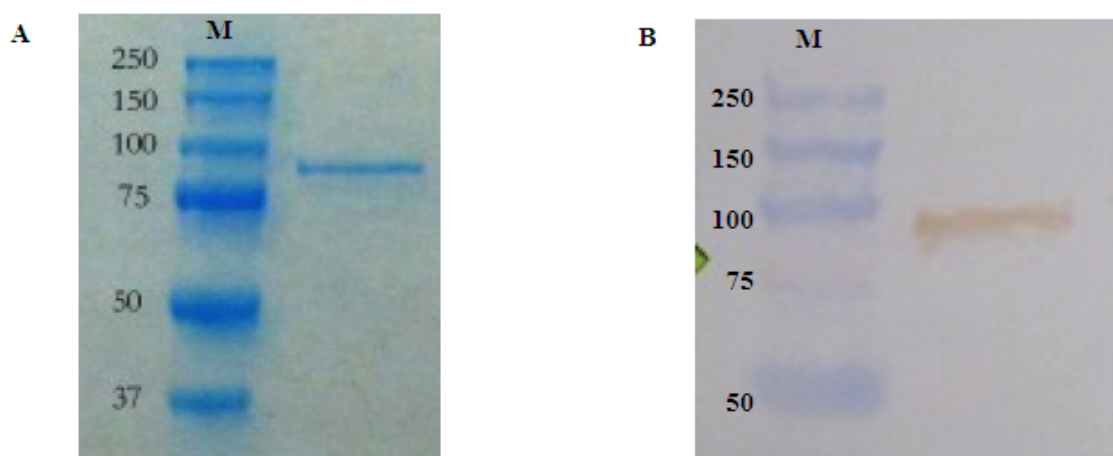
Appendices

Appendix I

Protein analysis of recombinant DnaK producing from *E.coli*

The recombinant DnaK was produced then it was stored at -80 °C as aliquots. The recombinant DnaK applied in every experiment of this thesis are identical. Before use, the recombinant DnaK was analyzed with SDS-PAGE, Western Blot and endotoxin test.

1µg DnaK was loaded in each well in SDS-PAGE. A Single band approximately 83kDa appeared on 10% SDS-PAGE (Fig. A). The increase in molecular mass relative to the native protein was due to the amino-terminal incorporation of thioredoxin encoded by the TOPO® cloning vector. The same 83kDa protein was visible on Western Blots probed with monoclonal antibody. (Fig. B). The endotoxin test was performed and the endotoxin content of the recombinant DnaK stock is 0.45 EU/mg DnaK.

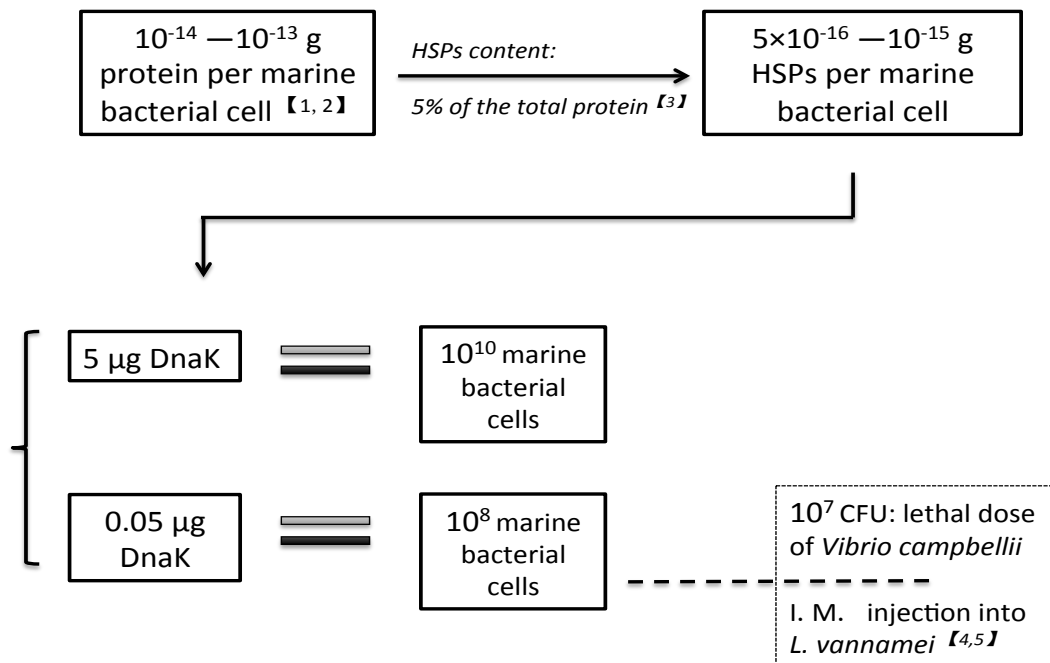


Recombinant DnaK produced in *E.coli*. 1µg Pure DnaK was resolved in SDS_PAGE gels and then either stained with Commassie Biosafe (A) or transferred to polyvinylidene fluoride membranes and probed with anti-DnaK antibody (B). Molecular mass standards (M) in kDa are on the left.

Appendix II

The calculation of DnaK dosage

In chapter 3 and chapter 4, two dosages of the recombinant DnaK were applied. The calculation of these two dosages is demonstrated here.



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Appendix III

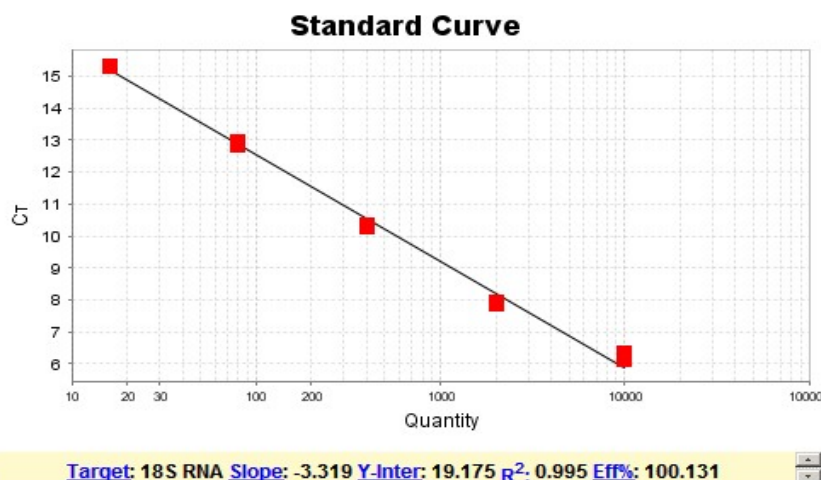
Reference gene selection in qPCR analysis: beta-actin or 18s RNA?

Quantitative real-time polymerase chain reaction (qPCR) is a useful technique to measure gene expression levels due to its high sensitivity, accuracy, and reproducibility. To employ qPCR for gene expression analysis, reference genes are used as internal control to normalize expression levels of other genes of interest. Therefore, selecting appropriate reference gene or adopting multiple reference genes is important to avoid analytical errors resulting from different experimental conditions or biological samples.

In this thesis, started from chapter 3 and chapter 4, another widely used reference gene, 18s RNA, were tested as well. Specific primers were designed and showed blow:

Gene	Genebank accession no.	Primer
18s RNA	AF186250.1	F: CGCTCGTAGTTTGACTTCTG R: CGACCATTTCGGGCTGTAA

The amplify efficiency of 18s RNA were measured by making a standard curve. Serial dilutions (5x) of the cDNA sample were made. Ct values of each dilution were measured in three replicates. The result showed that the amplify efficiency of 18s RNA was around 100% as same as other target genes.

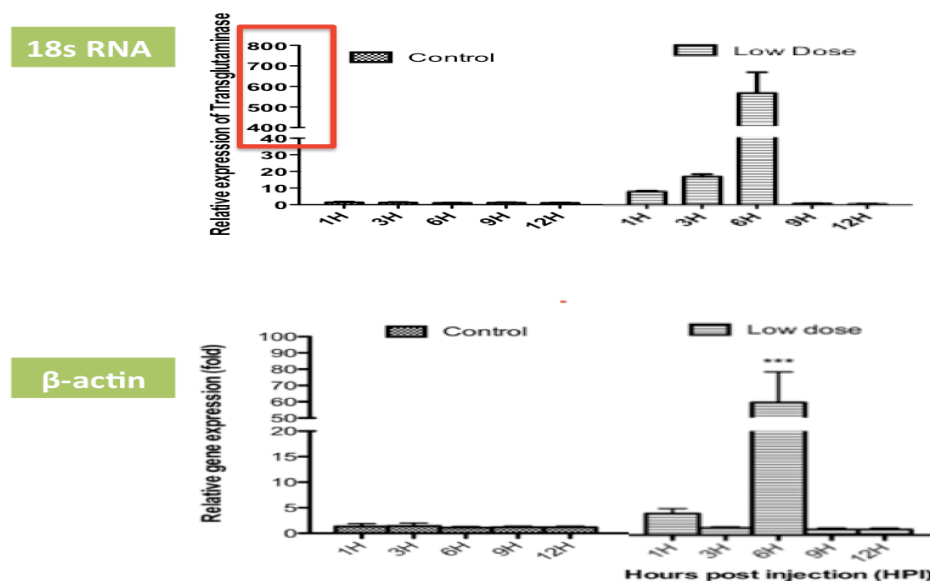


Appendices

However, the 18s RNA was not adopted into the qPCR analysis because of two reasons:

Firstly, its high abundance compared to the target gene transcripts. The Ct value of the 18s RNA varied from 6-10, while the Ct values of target gene, such as prophenoloxidase-2, transglutaminase-1, are around 20-30. It suggests that the 18s RNA content is much more than target genes in our RNA samples. The high abundance of 18s RNA makes it difficult to subtract the baseline value in real-time PCR data analysis.

Secondly, using 18s RNA as reference gene cause huge increases in fold number (showed in red box). It causes difficulties to present the data.



Therefore, the beta-actin was adopted as the reference gene in our study. Nevertheless, increasing studies have shown that only one reference gene is not sufficient to ensure an accurate qPCR analysis. More research needs to be done in the future about including multiple reference genes and their validation under specific biological condition.

SUMMARY

Summary

Summary

Aquaculture is the fastest growing animal food-producing sector in the world. According to the FAO global aquaculture production statistics for the year 2011, the total aquaculture production is estimated to be 62.7 million tons with an estimated value of US\$ 130 billion. Disease outbreaks are considered to be the main constraint to aquaculture development. The disadvantages of widespread use of antibiotics have been realized in whole animal production industry including aquaculture. Thus there is increasing demand for developing alternative disease control strategies.

The heat shock proteins (HSPs) were discovered in 1962. It has been a long research history discovering the heat shock response, molecular chaperone function and their distinct role in the immune modulation. Many studies have unveiled mechanisms of HSPs enhancing both innate and adaptive immunity within vertebrates. In vertebrates, even though knowledge about HSPs immune functions is very limited, several studies have reported an immune protective capability of HSP70 in crustaceans. It makes HSP70 become a potential candidate for the development of a new disease control approach. In this thesis, we aimed at verifying the immune stimulatory effect of a prokaryotic HSP70, DnaK, in *Litopenaeus vannamei* and subsequently evaluated if it is able to prime the immune system against *Vibrio campbellii* and White Spot Syndrome Virus (WSSV) infection by looking at the expression of immune-related genes. In Chapter 2, an overview of the recent research progress was made, which consists of two parts: crustaceans (shrimp) immune characters and HSPs immune functions.

In the first experimental study (Chapter 3), we aimed at verifying the role of DnaK as an immune activator in specific-pathogen-free (SPF) *L. vannamei* either by injecting them with recombinant full length DnaK or a chemically synthesized 50 amino acid fragment peptide which is part of the peptide binding domain of DnaK (DnaK₄₄₂₋₄₉₁). The expression of two types of immune-related genes (transglutaminases and prophenoloxidasases) were monitored *via* quantitative real time PCR (qRT-PCR). The results showed that DnaK and DnaK₄₄₂₋₄₉₁ are able to significantly up-regulate transglutaminase-1 (TGase-1) and prophenoloxidase-2 (proPO-2) expression within 12 hours post injection (hpi).

Summary

The synthetic peptide DnaK₄₄₂₋₄₉₁ is free of lipopolysaccharide (LPS) or any other bacterial components potentially contaminating a recombinant DnaK preparation. Hence the data suggested that DnaK, as such, stimulates the immune system of *L. vannamei* in a specific way (proPO-2, TGase-1). These findings indicate that DnaK is an efficient immune stimulator in *L. vannamei* immunity.

In chapter 4, the dynamics of serine proteinase (SP), prophenoloxidase (proPO), transglutaminase (TGase), penaeidins (PENs) and endogenous HSP70 (lvHSP70) gene expression were monitored *via* qRT-PCR. The SP, penaeidin-3 (PEN-3) and lvHSP70 were not significantly induced by the DnaK injection. However, the TGase-1 was dramatically up-regulated by a low dose of DnaK (0.05 µg/shrimp) to 60-fold at 6 hpi. The proPO-2 was induced 5-fold at 3 hpi both by the high dose and low dose of DnaK. The penaeidin-2 (PEN-2) and penaeidin-4 (PEN-4) expression slightly decreased at 6 hpi and then increased. Those results showed that two genes (proPO-2 and TGase-1) might be used as marker genes to study the host immune response to DnaK stimulation in *L. vannamei*.

In Chapters 5 & 6, the priming effect of bacterial HSP70 DnaK against *V. campbellii* and WSSV infection was evaluated. Chapter 5 demonstrated that pre-treatment of *L. vannamei* with the DnaK, followed by a non-lethal *V. campbellii* challenge (10⁵ CFU/shrimp) affected the transcription of 3 immune marker genes, TGase-1, proPO-2 and lvHSP70. The results showed that at 1.5 and 6 hour post DnaK priming (hpp) there is significant antagonistic interaction between DnaK and *V. campbellii* on the TGase-1 expression (two-way ANOVA, p<0.05). At 9 hpp, proPO-2 expression in the shrimps pre-treated with DnaK and then challenged with *V. campbellii*, displayed an additive effect, relative to the respective single treatments. However, a strong synergistic effect on lvHSP70 transcription was exhibited when shrimps were treated with both DnaK and *V. campbellii*. Taken together, we conclude that the DnaK can prime the expression of marker genes involved in the immune system of *L. vannamei* against *V. campbellii* infection.

A similar priming experiment was conducted in Chapter 6. The DnaK was used to prime the shrimp immune system, ahead of a challenge with the WSSV-

Viet strain which displays a reduced virulence relative to other WSSV strains. Transcriptions of two immune related genes, TGase-1 and proPO-2, were quantified within 12 hpp. Strong synergistic effects induced by both DnaK and WSSV on TGase-1 and proPO-2 were exhibited at 1.5 and 9 hpp respectively (two-way ANOVA, $p < 0.05$). These results indicate that the application of the DnaK may be considered as being part of an overall preventive strategy to fight WSSV in *L. vannamei*.

In conclusion, the results presented in this thesis indicate a possible role for DnaK as an immune priming agent in *L. vannamei* against *V. campbellii* and WSSV infections.

SAMENVATTING

Samenvatting

Samenvatting

Aquacultuur is de snelst groeiende sector met betrekking tot dierlijke productie. Volgens de FAO statistieken voor het jaar 2011, staat de teller van globale aquacultuur productie op 62.7 miljoen ton met een geschatte waarde van 130 miljard dollar. Ziekten worden beschouwd als een belangrijke belemmering voor de verdere ontwikkeling van aquacultuur. De nadelen verbonden aan het gebruik van antibiotica worden ook in de aquacultuur productie onderkend. Er is daardoor een toenemende vraag naar alternatieve methoden om ziekte controle strategieën te ontwikkelen.

Hitteschokproteinen (Hsp) werden ontdekt in 1962. Door volgehouden onderzoek werd hun belang in hitteschok resistentie, hun functie als moleculaire chaperonen en hun immuun-modulerende functie ontdekt. In gewervelden werden mechanismen beschreven die aantonen dat Hsp's de aangeboren en induceerbare immuniteit stimuleren. In ongewervelden, alhoewel op basis van een gelimiteerd aantal studies, werd aangetoond dat Hsp70 een beschermende capaciteit heeft. Hierdoor wordt Hsp70 beschouwd als een potentiële kandidaat in de ontwikkeling van nieuwe strategieën in ziektecontrole.

In deze thesis werd nagegaan in welke mate bacteriële HSP70, DnaK genaamd, een immuun-stimulerend effect heeft in *Litopenaeus vannamei*. Vervolgens werd bepaald of DnaK in staat is het immuunsysteem voor te bereiden op een *Vibrio campbellii* of White Spot Syndrome Virus (WSSV) infectie, door de expressie van immuun-gerelateerde genen te meten.

In Hoofdstuk 2 wordt een overzicht gepresenteerd van recente ontwikkelingen op het beschreven onderzoeksveld. Het bevat twee delen: garnaal immuun karakteristieken en HSP immuun functies.

In een eerste experimenteel gedeelte (Hoofdstuk 3) werd de rol van DnaK onderzocht met betrekking tot de mogelijkheid om het immuunsysteem te activeren, in specifiek pathogeen-vrije garnalen, door hen in te spuiten met recombinante volle-lengte DnaK of een synthetisch 50 aminozuur peptide dat deel uitmaakt van het peptide bindingsdomein (DnaK₄₄₂₋₄₉₁). De expressie van twee types van immuun-gerelateerde genen (transglutaminases and prophenoloxidasen) werd nagegaan via kwantitatieve PCR (qRT-PCR). De

Samenvatting

resultaten toonden dat DnaK en DnaK₄₄₂₋₄₉₁ in staat zijn transglutaminase-1 (TGase-1) en prophenoloxidase-2 (proPO-2) expressie te activeren binnen 12 uur na injectie. Het synthetisch peptide DnaK₄₄₂₋₄₉₁ is vrij van lipopolysaccharide (LPS) of enig ander bacteriële component. Die data tonen aan dat DnaK op zich het immuunsysteem stimuleert op een specifiek wijze (proPO-2, TGase-1) en dat het een efficiënte immuun-stimulerende werking heeft in *L. vannamei*.

In hoofdstuk 4 wordt de dynamiek van serine proteinase (SP), prophenoloxidase (proPO), transglutaminase (TGase), penaeidins (PEN) en endogenous HSP70 (lvHSP70) gene expressie opgevolgd via quantitative PCR. Het serine proteinase (SP), penaeidin3 (PEN3) and endogenous HSP70 (lvHSP70) werden niet significant geïnduceerd door DnaK injectie. Daarentegen, het transglutaminase-1 (TGase-1) werd zeer sterk opgereguleerd (60-voud) door een lage dosis van DnaK (0.05 µg/shrimp) op 6 hpi (uur post injectie). Het prophenoloxidase-2 (proPO-2) werd 5-voudig geïnduceerd 3 hpi door zowel een hoge als lage dosis van DnaK. De PEN2 and PEN4 expressie verlaagde lichtjes na 6 hpi en verhoogden dan opnieuw. Deze resultaten toonden aan dat 2 genen (proPO-2 and TGase-1) kunnen gebruikt worden als merker genen voor de studie van immuun response door DnaK injectie in *L. vannamei*.

In hoofdstuk 5&6 werd het immuun-voorbereidend effect van DnaK injectie op *Vibrio campbellii* and WSSV onderzocht. Hoofdstuk 5 toonde aan dat voorbehandeling van *L. vannamei* met DnaK, gevolgd door een niet-lethale blootstelling aan *V. campbellii* (10⁵ CFU/garnaal) de expressie beïnvloedde van 3 immuun marker genen, transglutaminase-1 (TGase-1), prophenoloxidase-2 (proPO-2) en endogene HSP70 (lvHSP70). De resultaten toonden een significante antagonistische interactie aan op 1.5 en 6 hpi op TGase-1 expressie (two-way ANOVA, $p < 0.05$). Op 9 hpi, proPO-2 expressie in DnaK voorbehandelde garnalen vertoonde een additief effect na *Vibrio* behandeling relatief ten aanzien van de enkelvoudige behandelingen. Daarentegen werd een sterk synergistisch effect op lvHSP70 transcriptie opgemeten wanneer garnalen werden behandeld met DnaK en *Vibrio*. Er werd besloten dat DnaK de expressie van immuun marker genen kan voorbereiden op een *Vibrio* infectie.

Een gelijkaardig experiment op een voorbereidend effect werd uitgevoerd in Hoofdstuk 6. DnaK werd gebruikt om het immuunsysteem voor te bereiden op

een blootstelling aan de WSSV-Viet stam, die een verlaagde virulentie vertoont ten aanzien van andere WSSV stammen. De transcriptie van transglutaminase-1 (TGase-1) en prophenoloxidase-2 (proPO-2) werd opgevolgd binnen 12 hpi na DnaK blootstelling. Een sterk synergistisch effect, geïnduceerd door DnaK en WSSV, op TGase-1 en proPO-2 expressie werd opgetekend op 1.5 and 9 hpi ($p < 0.05$). Deze resultaten toonden aan dat de applicatie van DnaK (de bacteriële HSP70 homoloog) kan beschouwd worden als een mogelijke strategie om WSSV in *L. vannamei* te bestrijden.

In conclusie, de resultaten voorgesteld in deze thesis, toonden een mogelijk rol aan voor DnaK als immuun-voorbereidend product in *L. vannamei* tegen *Vibrio* en WSSV infectie.

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Curriculum Vitae

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PUBLICATIONS

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1st International Conference of Fish and Shellfish Immunology. 2013. Vigo. Spain. Oral presentation.

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Gent